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(54) Title: SUBSTANCES HAVING THE GROWTH-PROMOTING EFFECT OF AMYLOID PRECURSOR PROTEIN

(57) Abstract

The invention includes an isolated peptide smaller than a native amyloid precursor protein (APP) that retains at least some neuronal growth promoting effect of APP. The peptide includes at least five consecutive amino acid residues with side-chain polarities corresponding to the side-chain polarities of the sequence RERMS. Non-peptide corresponding compounds and methods of obtaining these are also included. Moreover, the invention includes methods of treatment of neurological conditions using the peptides and non-peptides.

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SUBSTANCES HAVING THE GROWTH-PROMOTING EFFECT OF AMYLOID PRECURSOR PROTEIN

Field of the Invention

The present invention relates to the field of proteins associated with neuronal degeneration and growth. More particularly, the present invention relates to short peptide sequences that possess homology with regions within the secreted form of the amyloid ß-protein precursor compound that does not contain a Kunitz-type protease inhibitor domain (sAPP-695) that appears largely responsible for APP's growth promoting effects.

Background of the Invention

Alzheimer's Disease, herein "AD", is a central nervous system degenerative disease that involves the formation of amyloid or neuritic plaques. Post mortem brain tissue studies of AD confirmed patients show at least two other degenerative symptoms in addition to the amyloid or neuritic plaques: intraneuronal granulovacuolar degeneration, and neurofibrillary tangle formation. Several other neurodegenerative diseases of the central nervous system exhibit amyloid plaque formation, for instance, senile dementia, elderly Down's syndrome patients, and to a lesser extent, Pick's disease. Additionally, there are several hereditary amyloidoses where neuritic plaque formation is observed upon autopsy. For example, hereditary cerebral hemorrhage amyloidosis Dutch type (HCHWA-D), or Icelandic type (HCHWA-I). There are also certain so-called sporadic cerebral amyloid angiopathies (CAA's), as well as transmissible spongiform encephalopathies, and age-related amyloidoses (ARA's) that exhibit neuritic plaque formation at various stages of their pathologies.

The amyloid proteins are a major component of the cerebrovascular amyloid deposits, Glenner, et al., Biochem. Biophys. Res. Commun., 122:1131-1135 (1984), and the core of neuritic plaques, Masters, et al., EMBO J., 4:2757-2763 (1989); Selkoe, et al., J. Neurochem., 146:1820-1834 (1986), the latter of which is believed to be the hallmark of the

pathology found in the brain tissue of patients afflicted with Alzheimer's disease (AD). It is believed that the central pathological theme exhibited by these diseases is an apparently irreversible central nervous system neuronal degeneration or cell death.

There are also a variety of other conditions in which central nervous system neurons undergo degeneration. For example, ischemia, caused by physical or chemical assaults to the central nervous system, hypoxia, where oxygen is cut off from the tissue by either chemical or physical intervention, and free radical attack on the tissues, apparently due to metabolism of various chemicals or the normal aging process, to name but a few.

In the plaque producing diseases, such as AD and the progeny discussed above, the primary plaque component in each has been identified as a protein subunit of about 4.5 kD having the ability to aggregate. This protein subunit is variously referred to as the amyloid ß-protein, β-amyloid protein, or as amyloid A4, and is herein referred to as "A4." A4 is believed to be a degradation product from β-amyloid precursor protein (as used herein, "APP"). Kang, et al., Nature, 325:733-736 (1987).

APP appears as a triplet of polypeptides of molecular weight 110 to 130 kD on Western Blot and in immunoprecipitation. Siman, et al., J. Bio. Chem., 265: 3836-3843 (1990). APP is a membrane-spanning protein, (Kang, et al., Nature, 325:733-736 (1987); Tanzi, et al., Science, 235:880-884 (1987); Robakis, et al., Proc. Natl. Acad. Sci. USA, 84:4190-4193 (1987); Goldbager, et al., Science, 235:877-880 (1987)), of which at least five different forms of primary translation products are now known. Three forms (APP-563, APP-751 and APP-770) contain a domain showing a strong homology with protease inhibitors of the Kunitz type (KPI) (Ponte, et al., Nature, 331:525-527 (1988); Tanzi, et al., Nature, 331:528-530 (1988); Kitaguchi, et al., Nature, 331:530-532 (1988); De Sauvage, et al., Science, 245:651-653

(1989); Golde, et. al., Neuron, 4:253-267 (1990)) while the other two forms (APP-695 and APP-714) do not.

Subsequent studies have shown the existence of secreted forms of APP (sAPP), either in the medium of cultured cells such as PC12 and fibroblasts (Schubert, et al., Neuron, 3:689-695 (1989); Uéda, et al., Ann. Neurol., 25:246-251 (1989); Weidemann, et al., Cell, 57:115-126 (1989)) or in the cerebral spinal fluid (Palmert, et al., Proc. Natl. Acad. Sci. USA, 86:6338-6342 (1989); Weidemann, et al., Cell, 57:115-126 (1989); Kitaguchi, et al., Biochem. Biophys. Res. Commun., 166:1453-1459 (1990)). Indeed, each of the two major forms of APP is processed to form the two major secreted forms. The secreted form of the 695 amino acid protein is referred to herein as "sAPP-695". Further, protease nexin-2, referred to herein as "PN-2", has been identified as the major secreted form of the 751 amino acid translation product which contains a KPI domain. (Van Nostrand, et al., Science, 248:745-748 (1989); Oltersdorf, et al., Nature, 341:144-147 (1989)).

Since PN-2's identification as a form of APP, many reports have appeared describing biological functions for these forms of sAPP. These include the role in regulation of neurite extension (Van Nostrand, et al., Science, 248:745-748 (1989); Oltersdorf, et al., Nature, 341:144-147 (1989)), the role in blood coagulation process (Cole, et al., Biochem. Biophys. Res. Commun., 170, 288-295 (1990); Van Nostrand, et al., 1990; Smith, et al., Science, 248:745-748 (1990)) and the role in wound-healing process (Cunningham, et al., Brain Res. Rev., 16:95-96 (1991)). Little is known, however, about the physiological function of sAPP-695 which lacks KPI domain, in spite of the evidence indicating the APP-695 is the major form in the brain (Neve, et al., Neuron 1:669-677 (1988); Ponte, et al., Nature, 331:525-527 (1988); Tanaka, et al., Biochem. Biophys. Res. Commun., 157:472-479 (1988); König, et al., Mol. Brain Res., 9:259-262 (1991)).

The causes of A4 deposition, and the disease states associated with it, remain unknown. What is known is that through some mechanism A4 becomes irreversibly deposited

within the brains of certain individuals. It is believed that such deposition acts to compress neurons and lead to cell degeneration. Further, while, the causes of ischemic conditions, hypoxia, and free radical attack on tissues may often be known, traced, and/or linked to certain causes or events, neurons when subjected to these foregoing conditions will degenerate and die. When neurons undergo degeneration, generally, they will not self-repair.

It has been shown in recent studies that there appears to be a delicate balance between PN-2 and APP. For example, it is indicated that neuronal death in AD might be associated with a relative decline in sAPP-695 as compared to PN-2. Johnson, et al., Science, 248:854-857 (1990); Tanaka, et al., Biochem. Biophys. Res. Commun., 157:472-479 (1988). Moreover, it has been shown that a relative increase of PN-2 in relation to sAPP-695 is manifested in transgenic animals as AD type pathology. Quon, et al., Nature, 352:239-241 (1991).

In addition, a relatively increased level of PN-2 to sAPP-695 has been observed in aged rats, Neve, et al., Neuron, 5:329-338 (1990), and behaviorally impaired old rats, Higgins, et al., Proc. Nat'l Acad. Sci. USA, 87:3032-3036 (1990). However, some conflicting data exists with regard to this observation. Golde, et al., Neuron, 4:253-267 (1990); Konig, et al., Mol. Brain Res., 9:259-262 (1991).

We have also shown that sAPP-695 is secreted from cells and has a growth promoting effect on fibroblasts. Saitoh, et al., Cell, 58:615-622 (1989).

Thus, a need remains in the art for peptides or drugs to promote the healing of neurons that have undergone degeneration. Moreover, a need remains to prevent pathological changes to neurons that are caused by the above-mentioned diseases, as well as others.

Summary of the Invention

One aspect of the present invention provides an isolated peptide smaller than a native amyloid precursor protein (APP) that retains at least some neuronal growth promoting effect of APP, comprising at least five consecutive amino acid residues

with side-chain polarities corresponding to the side-chain polarities of the sequence RERMS (SEQ ID NO:1). In one embodiment, the peptide includes the sequence REKVT (SEQ ID NO:18). In another embodiment, the peptide includes the sequence RERMS (SEQ ID NO:1). Preferably, the peptide has 316 amino acid residues or less, still more preferably it has 150 amino acid residues or less, even more preferably 40 amino acid residues or less, even more preferably seventeen amino acid residues or less, and most preferably five amino acid residues or less. Thus, particularly preferred embodiments include a 40-mer corresponding to residues 296-335 of APP, a 17-mer corresponding to residues 319-335 of APP, and a 5-mer with side-chain polarities corresponding to the side-chain polarities of the sequence RERMS (SEQ ID NO:1). Thus, an especially preferred 5-mer corresponds to residues 328-332 of APP. Another aspect of the present invention provides a non-peptide drug effective in promoting neuronal growth. This non-peptide drug includes a plurality of functional groups. The functional groups have a polarity, electron distribution and bond length corresponding to the polarity, electron distribution and bond lengths of the side-chains in the peptide RERMS (SEQ ID NO:1). The drug can be produced by a method described below in which structural data is generated on a peptide that has neuronal growth-promoting activity and contains an amino acid sequence having a RERMS (SEQ ID NO:1) sequence or a RERMS-like sequence that has side-chain polarities corresponding to the side-chain polarities of the sequence RERMS, the peptide also containing a plurality of additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence located therein.

Thus, the invention also includes a drug comprising the non-peptide drug or the peptide as described above. The drug is capable of crossing the blood-brain barrier of the central nervous system of an animal. In one preferred embodiment, the peptide or non-peptide drug has a hydrophobic moiety attached to a terminal position of the drug in a manner such that the

hydrophobic moiety will not interfere with the activity of the drug.

Yet another aspect of the present invention provides a method of promoting the regeneration of damaged neurons *in vivo* in a mammal. This method includes identifying a mammal having damaged neurons, and administering a peptide or non-peptide drug as described above to the mammal. In a preferred embodiment, the damaged neurons are in the central nervous system (CNS) of the mammal.

The invention also provides a method of treating a condition associated with cerebral deposition of the amyloid β-protein in a human patient. This method includes identifying a patient having the condition, and administering a peptide or non-peptide drug as described above to the mammal. In one embodiment, the condition is Alzheimer's Disease (AD), and preferably, the patient has not yet developed complete Alzheimer's Disease symptoms.

In a further aspect, the invention provides a method of treating a neurological condition characterized by damage to neurons. Many such conditions can be treated, including ischemic neurological damage, hypoxic neurological damage, denervation following injury or trauma to the CNS and glutamate toxicity. The method includes identifying a patient having the neurological condition, and administering a peptide or non-peptide drug as described above. In a preferred form of the method, a neuroprotectant drug is also administered to the patient. The neuroprotectant drug can be any of many such drugs, including MK 801, other glutamate antagonists, an inhibitor of calpain, chlorpromazine and trifluoperazine.

The invention includes the peptide or non-peptide drugs described above for use as an agent to promote neuronal regeneration of neurons of the central nervous system *in vivo* in an animal, including the use for increasing the memory-retention ability of a mammal. The peptide or non-peptide is also provided for use as an agent to promote neuronal regeneration of central nervous system neurons in patients who

have been subject to an ischemic or hypoxic event.

Yet another aspect of the present invention is a method of increasing the memory-retention ability of a mammal. In this method, a peptide or non-peptide drug as described above is administered to the mammal in a manner such that the peptide or non-peptide enters the central nervous system of the mammal. The peptide or non-peptide can be administered by any of a variety of administration routes, including intramuscular injection, intravenous injection, intrathecal injection, direct infusion into the central nervous system and oral administration.

Still one more aspect of the invention provides a growth assay for testing the neuronal growth-promoting activity of a chemical substance. This assay includes the step of adding a measured quantity of a the substance, to a culture of a fibroblast cell line that is deficient in its secretion of APP. The cell line should have a substantially slower growth rate than a normal fibroblast cell line not having the deficiency. The assay then includes measuring the growth rate of the culture containing the substance and measuring the growth rate of the culture in the absence of the substance. The growth rate of the culture containing the substance with the growth rate of the culture in the absence of the substance is then compared. An increase in the growth rate of the culture containing the substance relative to the growth rate of the culture in the absence of the substance indicates that the substance has neuronal growth-promoting activity. Although many cell lines can be used, the fibroblast cell line A-1 derived from parent cell line AG2804 is one preferred cell line for use in the assay. Cell line B103 is another such preferred cell line.

As discussed above, the invention includes a method of designing non-peptide drugs for use as neuronal growth promotion agents. This method includes generating structural data on a peptide that has neuronal growth-promoting activity and contains an amino acid sequence having a RERMS (SEQ ID NO:1) sequence or a RERMS-like sequence that has side-chain

polarities corresponding to the side-chain polarities of the sequence RERMS, the peptide also containing a plurality of additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence located therein. The method also includes determining energetically stable conformations of the peptide by comparing the structural data to structural data of a model protein or peptide using molecular dynamics calculations. The model peptide protein has substantial homology to SEQ ID NO:8. Further steps of the method are determining the active domain in the peptide, calculating the electrostatic potential of the active domain, and designing a low molecular weight non-peptide compound that has a structure that has an energetically stable conformation and matches the electrostatic potential, as determined in the foregoing steps. Finally, the non-peptide compound is synthesized. The step of determining the active domain can be performed by producing variants of the peptide having an amino acid substitution in the RERMS or RERMS-like sequence thereof, such as in the additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence. Thus, this determining step can also include determining which of the variants provide neuronal growth-promoting activity. In a preferred form of this method, the structural data of the peptide is generated by a method such as nuclear magnetic resonance (NMR), X-ray crystallography, circular dichroism, or small angle neutron scattering (SANS). More preferably, the generating step can preferably include the use of NMR. The comparing step can use glucose-6-phosphate isomerase as the model compound. In the step of determining the active domain, active and inactive substitution parameters can be derived from a growth assay as is described above. The calculating step can include the use of a calculation protocol such as MNDO, AMI or PM3. The designing step can include the steps of obtaining relative configurations of the RERMS or RERMS-like sequence and maintaining the relative configuration in order to design a hydrocarbon or substituted hydrocarbon skeleton

that will maintain such relative configuration of amino acids, substituting homologous model moieties onto the skeleton for the original amino acids to produce a model molecule, calculating the structure and distribution of electrons on the model molecule to produce a structural and distributional result, comparing the structural and distributional result with that for a known molecule that has been previously modeled, repeating the substituting, calculating and comparing steps for a plurality of homologous model moieties, determining the optimum homologous moiety for replacement of the homologous model moieties, and synthesizing the molecule that comprises the optimum homologous moieties substituted onto the skeleton. The method of Claim , wherein the non-peptide drug is also made capable of crossing the blood-brain barrier in the central nervous system of an animal, such as by adding a hydrophobic moiety to a terminal position of the drug and in a manner such that said hydrophobic moiety will not interfere with the activity of said drug.

Still a further aspect of the present invention provides a pharmaceutical composition for the treatment of a medical condition associated with neuronal degeneration in the central nervous system of a mammal. This composition includes a peptide or non-peptide drug as described above in an amount effective to promote the growth or regeneration of neurons *in vivo* together with a pharmaceutically acceptable carrier, filler, or excipient. The drug can be present in the composition in an amount effective to treat a condition associated with cerebral deposition of amyloid β-protein, a hypoxic condition of neurons in the central nervous system, an ischemic condition of neurons in the central nervous system and a condition affecting memory retention. In certain preferred forms of the composition, the composition also includes a neuroprotectant drug, such as MK 801, other glutamate antagonists, an inhibitor of calpain, chlorpromazine and trifluoperazine.

Still one more aspect of the invention provides a

pharmaceutical composition for increasing the memory-retaining ability of a mammal. In this aspect, the composition includes a peptide or non-peptide drug as described above in an amount effective to increase the memory-retaining abilities of the mammal together with a pharmaceutically acceptable carrier, filler, or excipient.

The invention also includes an antagonist of the neuronal growth-promoting activity of amyloid precursor protein, such as a peptide containing the sequence RMSQ (SEQ ID NO:2).

Brief Description of the Drawings

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Description of the Preferred Embodiments

In the ensuing detailed description reference to the standard single-letter amino acid abbreviations are used. See, e.g., "Short Protocols in Molecular Biology," eds. Ausubel et al., Greene Publishing Associates (1989), p. 359.

We have recently discovered that only a small portion of the amino acid sequence of sAPP-695 is responsible for the growth enhancement in fibroblasts. Roch, et al., J. Biol. Chem., 267:2214-2221 (1992). We have further discovered a series of fragments of sAPP-695 that are uniquely responsible for the growth promoting effects of sAPP-695 in neurons. Surprisingly, we have discovered that only a 5 amino acid sequence on APP are necessary to achieve the growth promoting effect of APP, or, in other words, it is a 5-mer sequence, corresponding to residues 328-332 of APP, or R-E-R-M-S or Arg-Glu-Arg-Met-Ser (SEQ ID NO:1), that is responsible for the growth promoting effect of APP. Moreover, in our studies that led to this discovery, we isolated and found growth promoting activity in several peptides that include the 5-mer sequence. Such fragments include a 40-mer, consisting of 40 amino acids, residues 296-335 of APP, and a 17-mer, consisting of 17 amino acids, residues 319-335 of APP and an 11-mer, consisting of 11 amino acids, residues 325-335 of APP. See, e.g., Kang et al. *supra* for the full sequence of APP. Each of the fragments within the series possesses the 5-mer sequence of R-E-R-M-S

(SEQ ID NO:1). Moreover, the 5-mer possesses a substantial amount of the neuronal growth promoting activity as native sAPP-695, however, less activity than either the 40-mer, the 17-mer or the 11-mer.

Indeed, the sequence of five amino acids, RERMS (SEQ ID NO:1) (APP328-332), is uniquely required for the growth-promoting activity of sAPP-695 on fibroblasts. For example, in studies that we have conducted, peptides of APP in which the RERMS (SEQ ID NO:1) sequence is deleted or reversed do not possess growth promoting activity. Moreover, we also found that the four amino acid peptide RMSQ (SEQ ID NO:2), which corresponds to residues 330-333 of APP, and which partially overlaps the C-terminal side of the active sequence RERMS (SEQ ID NO:1), acts to antagonize the activity of sAPP-695. These findings firmly established the presence of biological activity of sAPP-695 which lacks the KPI domain.

Peptides which contain the active sequence RERMS (SEQ ID NO:1) of the present invention are useful to promote the growth of neurons *in vitro* and *in vivo*. We have promoted the growth of fibroblasts which are deficient in their secretion of APP and, consequently, exhibit profoundly slow growth. Such fibroblast's growth, however, may be promoted by addition of exogenous APP. Profoundly, the peptides that we have discovered that possess the RERMS (SEQ ID NO:1), promote growth of the A-1 cell line. We have also promoted the growth of neurons, both *in vitro* and *in vivo*. For example, we have induced ischemic conditions into rabbits through cutting off aortic blood flow, followed by infusing the rabbits' spine with an APP moiety with the RERMS sequence (SEQ ID NO:1). Neurite growth is observably increased and the animals' functions appear to return to normal.

In the following discussion, we shall discuss the characterization and fixation of the active growth promoting site of APP, the treatment of neurons with APP moieties containing RERMS sequence (SEQ ID NO:1), and the design of synthetic drugs to mimic APP moieties containing the RERMS

sequence (SEQ ID NO:1).

A. Characterization and Fixation of the Growth Promoting Activity of APP

The above discoveries arose in connection with our attempts to study physiological functions of APP. We have previously disclosed a fibroblast cell line A-1, transfected with a plasmid expressing an antisense APP RNA. See Saitoh, et al., Cell, 58:615-622 (1989) which is incorporated herein by reference in its entirety. The A-1 cell line produces very low levels of APP mRNA and its translation products, resulting in a slow growth rate. The growth of A-1 cells, however, can be restored to the level of the normal or parent fibroblasts cell line, AG2804, by addition of exogenous APP, either APP-695 or APP-751/APP-770, into the culture medium. Saitoh, et al., Cell, 58:615-622 (1989); Bhasin, et al., Proc. Natl. Acad. Sci. USA, 88:10307-10311 (1991).

Although the biochemical basis for the retarded growth rate of A-1 cells is still unclear, the dependence of the growth of cells on exogenous APP provide a rather simple bioassay to study the functional mapping of APP. Roch, et al., J. Biol. Chem., 267:2214-2221 (1992).

Consequently, through use of the A-1 cell line, we were able to test the activity of a series of sAPP-695 fragments obtained from our bacterial expression system. In this way, we discovered and recently disclosed that at least one of the active sites of sAPP-695 was localized within a 40-mer sequence of APP, within residues 296-335 of the Kang sequence. See Roch, et al., J. Biol. Chem., 267:2214-2221 (1992).

We obtained and maintained our cell lines in accordance with the following example.

EXAMPLE I
Cell Culture

As mentioned above, we use the cell lines A-1 and AG2804 (parent) fibroblasts for a simple bioassay of the growth promoting effect of our APP fragments. Such cell lines are prepared and maintained as described previously. See Saitoh, et al., Cell, 58:615-622 (1989). In brief, AG2804 cells were

cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in 75 ml flasks with one passage a week. A-1 cells were maintained in the same way with the exception that the media contained the conditioned media (CM) from AG2804 cell culture (20% v/v). Inclusion of the CM from AG2804 cells was necessary because the growth of A-1 cells was significantly slower than that of AG2804 cells and was restored by the addition of the CM. Saitoh, et al., Cell, 58:615-622 (1989); Bhasin, et al., Proc. Natl. Acad. Sci. USA, 88:10307-10311 (1991). To eliminate any residual effect of the CM, A1 cells used for the growth assay were kept in the regular media (DMEM/10% FCS) without CM for one week before the assay.

Since our discovery of the 40-mer, and in order to further characterize the growth-promoting activity of sAPP-695 on fibroblasts, we have addressed the following questions: (i) which amino acid sequence within the 40-mer is essential for the activity; (ii) is the activity sequence-specific; (iii) is the 40-mer sequence the only active site of sAPP-695; and (iv) does the activity arise through promoting the adhesion of the cells?

As was mentioned above, APP appears to possess sites that act as agonists and antagonists in neuronal growth promotion. This finding is useful in further delineating the biological activity of sAPP-695 in physiological and pathological conditions. In our earlier disclosure, Roch, et al., J. Biol. Chem., 267:2214-2221 (1992), we showed that both KB75 (APP residues 20-591) and 40-mer (APP296-335) were active in our A-1 cell growth assay. To find out the amino acid sequence within the 40-mer that was essential to its activity, we synthesized a battery of peptides and tested their activity on the A-1 cell growth assay.

The synthetic peptides were prepared in accordance with the following example.

EXAMPLE II

Preparation of Synthetic Peptides

Peptides corresponding to all or part of APP296-335 were

synthesized using a Rainin PS3 peptide synthesizer and purified on a C-18 reverse-phase HPLC column. The amino acid sequences of the synthetic peptides synthesized and used are detailed in Table I. Each peptide was recovered in 40% acetonitrile. The acetonitrile was eliminated by evaporation under vacuum, and the peptide was resolubilized in 50 mM $NaHCO_3$ (pH 7.4). Because both C6 and C7 peptides were insoluble in the buffer due to their hydrophobicity, they were first dissolved in dimethyl sulfoxide (DMSO) and then diluted to give the final concentration of DMSO less than 0.01% in the assay media. The presence of 0.01% DMSO in the media did not affect the growth of A-1 cells (data not shown). All the other peptides used were soluble in the sodium bicarbonate buffer. Each of the peptides that we synthesized are listed in Figure __.

We also engineered a plasmid KB75 that encodes for APP residues 20 through 591 and a plasmid KB75δ which encodes APP residues 20 through 591 without residues 306-335. The construct KB75δ was designed to enable us to see more directly whether the domain within residues 306 through 335 is the only active site of sAPP-695 or if there is an additional active site. As will be appreciated, this is enabled since residues 306 through 335 contains the majority of the 40-mer sequence and all of the 17-mer and 5-mer. Thus, since the 40-mer, 17-mer, and 5-mer are all known to be active, their elimination from KB75δ allows us to determine if there are other regions with growth promoting activity.

EXAMPLE III

Plasmid Construction and Purification of APP Construct KB75 and KB75δ

Each of the constructs KB75 and KB75δ were obtained through the bacterial expression and protein purification procedures, which are described in greater detail in Roch, et al., J. Biol. Chem., 267:2214-2221 (1992), in connection with KB75, which encodes for the amino acids Val-20 to Ile-591 of sAPP-695.

The strategy for the construction of the plasmid pKB75δ

is shown in Figure 7. The oligonucleotides used as primers in the PCR reaction were synthesized in a PCR-mate DNA synthesizer (Applied Biosystems) and purified by the OPC system. The sequence of the 5' primer was 5'-GACAGTGTCACTCGAGAGAGAATGGGAAGAGGCAGAA-3' (SEQ ID NO:3) and the 3' primer was 5'-GGACTGAGTCCTCGAGCTAGATCTCCTCCGTCTTGAT ATT-3'(SEQ ID NO:4). The template for the PCR reaction was the 2.4 kb SmaI-XmnI fragment of APP-695 cDNA. The reaction was carried out in 100 μl containing 500 ng of template DNA, 1 μM of each primer, 200 μM of each dNTP and 1.5 units of enzyme AmpliTaq. The sample was brought to a temperature of 94°C for 3 minutes, followed by 38 cycles, following the protocol: 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute. After the last cycle, a final extension step was performed at 72°C for 7 minutes.

The PCR product (800 bp in length) was finally purified by electrophoresis in 1% agarose gel, binding to a DEAE-cellulose membrane, elution in 1M NaCl at 65°C, and ethanol precipitation. The purified PCR product was then digested with XhoI restriction enzyme, and ligated into the plasmid pKB75δ previously digested with XhoI and treated with canine intestine alkaline phosphatase. Competent E. coli MC1061 cells were transformed with the ligation mixtures and clones were selected for ampicillin resistance. The size and orientation of the insert were determined by restriction analysis, using different enzymes having asymmetrical restriction sites.

Positive clones were tested for expression of APP-derived peptides of appropriate size by immunoblotting with anti-GID antibody as described below. The new plasmid was named pKB75δ.

EXAMPLE IV

Immunoblotting

We used anti-GId antibody which is a rabbit polyclonal antibody against the peptide 175-186 of APP (Saitoh, et al., Cell, 58:615-622 (1989)) to verify the proteins expressed in

bacteria. Proteins were separated by SDS-PAGE (8-16% Tris-glycine gel) and transferred onto a nitrocellulose filter. The filter was blocked 1 hour at room temperature in PBS containing 0.1% Tween 20 (TPBS), incubated overnight at 4°C in PBS containing 3% bovine serum albumin (BSA) and the antibody (1:2000), washed five times for 5 min in TPBS at room temperature, incubated 2 hours at 4°C in PBS containing 3% BSA and ^{125}I-protein A (Amersham Co., 0.3 μCi/ml), washed again as described above, and exposed onto Kodak RP film for 2 hours at room temperature.

Immunoblotting with anti-GID antibody detected the expression of KB75δ in the partially purified bacterial lysate, with a predicted difference in the molecular weight from that of KB75 (Fig. 4C). The peptide was further purified through heparin-agarose column and size-exclusion HPLC, to give an apparently single band on Coomassie blue staining of the SDS-PAGE gel (data not shown). When KB75δ thus verified was tested on A-1 cell growth assay, we could see no activity (Fig. 5), indicating that the sequence Thr-306 to Met-335 which was deleted in KB75δ was essential for the activity.

The resulting fragments from the above peptide syntheses were then screened for their growth promoting effects in connection with our bioassay with our fibroblast cell line A-1. Such screening was conducted in accordance with the following example.

EXAMPLE V

Growth Assays

Cells at 70-80% confluence were detached from the substratum by incubating in phosphate buffered saline (PBS) containing 1mM EDTA. The detached cells were harvested, centrifuged down and resuspended in DMEM/10% FCS at the density of 5,000 cells/ml. The cell suspension was then seeded in six-well plates (2 ml/well) and the plates were kept at 37°C in the CO_2 incubator for one hour. The relevant peptide solution in DMEM (100 μl) was then added. the same volume of DMEM without peptide was added in control wells.

The plates were kept at 37°C in the CO_2 incubator for three days and the cell number in each well was determined using a Coulter cell counter, model ZF (Coulter Electronics, Inc., Hialeah, FL). The results were expressed as the percent increase in the cell number compared to the number of the cells seeded. All the growth assays were done in triplicate.

With this growth assay, we were able to screen each of the peptides synthesized in accordance with Example II. We conducted our screening in accordance with the following Example to fix the precise location on APP for the growth promoting effect.

EXAMPLE VI

Fixation of the Location On APP of Growth Promoting Activity

We utilized the following protocol to determine where on the 40-mer the active sites were located. Starting with the 40-mer:

T-P-D-A-V-D-K-Y-L-E-T-P-G-D-E-N-E-H-A-H-F-Q-K-A-K-E-R-L-E-A-K-H-R-E-R-M-S-Q-V-M (SEQ ID NO:5)

We prepared three peptides which covered the N-terminal (14-mer), middle (13-mer) and C-terminal (17-mer) portions of the 40-mer:

T-P-A-V-D-K-Y-L-E-T-P-G-D (SEQ ID NO:6)

G-D-E-N-E-H-A-H-F-Q-K-A-K (SEQ ID NO:7)

A-K-E-R-L-E-A-K-H-R-E-R-M-S-Q-V-M (SEQ ID NO:8)

Each of these peptides were tested for activity in accordance with Example V and we determined that only the 17-mer possessed activity. See Figure 2.

Thus, we constructed an 11-mer and an 8-mer from the 17-mer to ferret out the areas of activity in the 17-mer:

```
    8-mer (SEQ ID NO:9)
  |-------------|
A-K-E-R-L-E-A-K-H-R-E-R-M-S-Q-V-M  (SEQ ID NO:8)
            |-------------------|
                  11-mer (SEQ ID NO:10)
```

Only the 11-mer showed activity. See Figure 3. Thus, we prepared a battery of peptides that possessed varying sequences within the 11-mer and activity for each was tested:

Peptide	Sequence	Sequence	Activity
N5	A-K-H-R-E	SEQ ID NO:11	(-)
N4	K-H-R-E	SEQ ID NO:12	(-)
M6	H-R-E-R-M-S	SEQ ID NO:13	(+)
M5	R-E-R-M-S	SEQ ID NO:1	(+)
M4	R-E-R-M	SEQ ID NO:14	(-)
M3	R-E-R		(-)
11-Mer	A-K-H-R-E-R-M-S-Q-V-M	SEQ ID NO:8	(+)
C7	E-R-M-S-Q-V-M	SEQ ID NO:15	(-)
C6	R-M-S-Q-V-M	SEQ ID NO:16	(-)
C4	R-M-S-Q	SEQ ID NO:2	(-)

First, we tested peptides that covered the N-terminal (N4 and N5), middle (M5 and M6) and C-terminal (C6 and C7) portions of 11-mer. Of those, only M5 and M6 were active, although the concentrations of the peptides required for significant growth effects were approximately tenfold higher than those of other active peptides described above, the 17-mer, 11-mer, and the 40-mer (Fig. 1C). Additionally, we synthesized and tested the peptides N4/N5 and C6/C7, none of which were active. See Figure 4.

The negative result for C7 (ERMSQVM (SEQ ID NO:15)) and C6 (RMSQVM (SEQ ID NO:16)) suggested that the N-terminal R and E residues of M5 (RERMS (SEQ ID NO:1)) were indispensable for the activity. Thus, to ascertain whether the C-terminal S and M residues were indispensable or not, we tested M4 (RERM (SEQ ID NO:14)) and M3 (RER) and found no activity (Fig. 5). This result suggests that both of the C-terminal M and S residues are required for the activity. Thus, we have narrowed down the active site to the 5 amino acids sequence RERMS (SEQ ID NO:1) (residues 328 through 332 of the Kang APP sequence).

EXAMPLE VII

Sequence Specificity of Activity

To test the sequence-specificity of the activity, we constructed and tested reverse-sequence 17-mer: M-V-Q-S-M-R-E-R-H-K-A-E-L-R-E-K-A (SEQ ID NO:17). The reverse sequence 17-mer was prepared in accordance with Example II and tested in accordance with Example V. As will be seen in Figure 3, no activity was detectable in the reverse sequence 17-mer.

EXAMPLE VIII

Cell Incorporation Studies

As an additional parameter of the growth of cells,

incorporation of tritiated thymidine into the cells was determined. The growth assay of Example V was followed, with the additional step of measuring the tritiated thymidine's incorporation. This was accomplished by washing the cells after three days incubation with DMEM and then incubating the cells in DMEM containing [^{3}H]thymidine (Amersham Co., 5 μCi/ml, 1 ml/well) for three hours at 37°C in the CO_2 incubator.

The cells were then fixed with methanol/acetic acid (1:3 v/v) and washed with ice-cold 10% TC (0.5 ml x 5 times). The radioactivity remained in the cells was extracted by overnight incubation in 0.5 ml of 1 N NaOH and was counted in 10 ml of scintillation cocktail.

We found that after three days exposure of cells to either KB75 or 17-mer (both at 10 pM) there was a significant increase in the incorporation of tritium. See Figure 6. This finding reflects an increase in the cell number per well, whereas neither 14-mer nor 13-mer caused the higher levels of [^{3}H]thymidine incorporation at the same concentrations, consistent with the results of cell numbers.

EXAMPLE IX

Antagonistic Action of Overlapping Peptide Fragments

One of the possible mechanisms for the action of these APP peptides is the presence of cell-surface molecule(s) or receptors recognized by this sequence. The sequence specificity of the activity, as described above in connection with Example VII, appears to support this theory. Accordingly, we expected that the inactive peptides which partially overlap the active sequence RERMS might have an antagonistic action against the active peptides. To determine if the overlapping peptides possessed any antagonistic action against the active peptides, we tested the activity of either 17-mer or KB75 (at 10 pM) in the presence of the peptides that did not show growth promoting activity themselves and which partially overlap the active 5-mer, RERMS (SEQ ID NO:1).

To accomplish this test, we utilized the growth assay in accordance with Example V, with the additional step of

including the respective inactive peptides with the active 17-mer or KB75 peptides. An excess of the inactive peptides was utilized (10 nM). We determined that both C7 and C6, but not N4, antagonized the activity of the peptides (Figure 7). Initially, we were concerned that the antagonistic action might have been an artifact caused by DMSO. However, this is not the case because C4 (RMSQ SEQ ID NO:2), which was a water-soluble derivative of C6, had a similar antagonistic action (Figure 7). C4 by itself had no growth-stimulating activity (data not shown).

The fact that the same peptide (C4) could antagonize the activities of either 17-mer or KB75 to a similar extent is indirect evidence which indicates that this domain within the 17-mer represented the only active site on the 40-mer.

EXAMPLE X

Experiments to Determine if There are Additional Regions of Growth Promoting Activity on APP

To see more directly whether the RERMS (SEQ ID NO:1) site is the only domain on sAPP-695 that contains growth promoting activity, or if there are additional active sites, we engineered a construct which encodes APP20-591 without residues 306-335 (pKB75δ) (Fig. 8, 9), in accordance with Example III, above.

Briefly, recombinant protein KB75δ was obtained through the bacterial expression and protein purification procedures, which were exactly the same as employed for the preparation of KB75. Roch, et al., J. Biol. Chem., 267:2214-2221 (1992). Immunoblotting with anti-GID antibody detected the expression of KB75δ in the partially purified bacterial lysate, with a predicted difference in the molecular weight from that of KB75.

The peptide was further purified through heparin-agarose column and size-exclusion HPLC, to give an apparently single band on Coomassie blue staining of the SDS-PAGE gel (data not shown). Through subjecting KB75δ to our growth assay, as discussed in connection with Example V, we found that KB75δ did not possess any growth promoting activity or effect with

the A-1 cell line. This result indicates that the deleted sequence Thr-306 to Met-335, in KB75δ, is essential for the activity of the APP and its fragments.

EXAMPLE XI

Heparin Binding Peculiarities

During the purification of KB75/KB75δ from bacterial lysate involving the use of heparin-agarose column, we unexpectedly noticed that the ionic strength of NaCl required for the elution of KB75δ was consistently lower than that required for the elution of KB75, suggesting that the deleted sequence of Thr-306 to Met-335 in KB75δ contained a heparin-binding domain of KB75, or alternatively, the deletion caused a significant change in the higher structure of the molecule resulting in a reduced heparin-binding affinity.

To see whether the deleted region represented a heparin binding site or caused a significant change in the higher structure of the molecule which then resulted in a reduced heparin binding affinity, we applied the synthetic peptides corresponding to the deleted sequence (i.e., the 40-mer and 17-mer) to the same columatography and found no heparin-binding capacity for the peptides (Figure 10). Thus, although the deleted sequence in KB75δ might contribute to the heparin-binding capacity of sAPP-695 in an unknown way, the biological activity of the active domain included in the deleted sequence apparently is not due to the heparin-binding.

EXAMPLE XII

Cell Adhesion Studies

To study the adhesion of the cells, we used the following assay. Cells were harvested, washed, and resuspended as described above, in DMEM/10% FCS at the density of 20,000 cells/ml. The cells were then seeded in six-well plates (2 ml/well) and the relevant peptide solution in DMEM (100 μl) was added immediately. The same volume of DMEM without peptide was added in control wells. The plates were kept at 37°C in the CO_2 incubator. After various lengths of time, the plates were subjected to rotatory shaking (100 rpm for 1 min). Both the number of floating cells recovered in the media and

the number of attached cells recovered by trypsinization of the well were determined by the Coulter counter.

In some experiments where the effect of immobilized peptide on the cell adhesion was tested, the wells were coated with the peptide according to the procedures described by Schubert, et al., Neuron, 3:689-694 (1989) and the adhesion of cells to the precoated wells was determined as described above. All the assays were done in triplicate. The results were expressed as the percentage of cells recovered in the media compared to the total number of cells recovered.

We used this cell adhesion assay as another attempt to find out the possible mechanism for the growth-promoting activity of sAPP to study the effect of KB75 on the adhesion of A-1 cells to the culture well. Although there was a significant decrease in the adhesiveness of A-1 cells compared to that of AG2804 cells, addition of KB75 did not help A-1 cell adhesion (Fig. 11). Other peptides tested (40-mer and 17-mer) gave similar negative results (data not shown). Coating the wells with KB75 (according to the procedure described by Schubert, et al., Neuron, 3:689-695 (1989)) prior to the assay did not help A-1 cell adhesion, either (data not shown).

The lack of the activity of KB75δ in the present study (Fig. 12) is a strong evidence indicating that the deleted sequence in KB75δ (APP306-335, Kang sequence) included the only one active site of sAPP-695 in the A-1 cell bioassay. The growth-promoting activity of this site was represented by the 5 amino acids sequence RERMS (APP residues 328-332) SEQ ID NO:1 (Fig. 1). The concentration of the peptide needed for the significant effect, however, was approximately tenfold higher for RERMS than for either the 11-mer or 17-mer, suggesting that the amino acid sequence in the vicinity was necessary for the full activity. As mentioned above, the reverse-sequence 17-mer had no activity (Fig. 3), further indicating the sequence-specificity of the activity. We also found that the 4 amino acids peptide RMSQ (SEQ ID NO:2)

(APP330-333), which partially overlaps the C-terminal side of the active sequence RERMS, could antagonize the activity of either 17-mer or KB75 (Fig. 8).

The RERMS (SEQ ID NO:1) motif and its N-terminal side amino acid sequence have some interesting characteristics. First, the sequence RERMS (SEQ ID NO:1) is unique for APP; we found no match in the peptide sequences so far registered in GeneBank. Second, it is within one of the evolutionary highly conserved regions of APP (Rosen, et al. 1989). Exactly the same sequence as 40-mer is found in mammalian APPs (rat, mouse, and monkey). In the Drosophila "APP-like" molecule, there is a sequence REKVT (SEQ ID NO:18) (residues 423-427; Rosen, et al. 1989); the polarity of the side chains of the corresponding residues is the same as RERMS (SEQ ID NO:1) (K and R have basic, V and M have nonpolar, and T and S have uncharged polar side chains, respectively). The uniqueness and the high evolutionary conservation together suggest the fundamental role of the sequence in the physiological function(s) of sAPP.

We could thus identify a unique region of APP which was active on our A-1 cell bioassay. The biochemical mechanism of the activity, however, is still unknown. As discussed in previous reports (Saitoh, et al. Cell, 58:615-622 (1989); Roch, et al., J. Biol. Chem., 267:2214-2221 (1992)), we proposed two possible mechanisms. The first is the possible effect of APP on the A-1 cell adhesion. Many reports have suggested the role of APP in cell adhesion. Schubert, et al., Neuron, 3:689-695 (1989); Breen, et al., J. Neurosci. Res., 28:90-100 (1991); Chen, et al., Neurosci. Lett., 125:223-226 (1991). APP binds to heparin-sulfate proteoglycan (Narindrasorasak, et al., J. Biol. Chem., 266:12878-12883 (1991)), which is supposed to be involved in cell-extracellular matrix interaction.

We thus examined the effects of sAPP peptides on A-1 cell adhesion and found that, although there was a significant reduction in the adhesiveness of A1 cells compared to that of the parent cells, AG2804, neither soluble nor immobilized sAPP

helped the adhesion of A-1 cells (Fig. 11). Because A-1 cells produce very low levels of both membrane-bound (intact) and secreted forms of APP (Saitoh, et al., Cell, 58:615-622 (1989)), these results may suggest that those involved in the adhesion of A-1 fibroblasts is not the secreted forms but the membrane-bound forms of APP and that the secreted forms of APP have a different mechanism of action for the growth-promoting activity.

The second possible mechanism we proposed is the effects of sAPP on the intracellular signal transduction machineries through an interaction with cell-surface receptor molecule(s). The sequence-specificity of the activity and the antagonistic actions of partially overlapping, inactive peptides are compatible with this notion.

APP is one of the heparin-binding proteins (Schubert, et al., Proc. Natl. Acad. Sci. USA, 86:2066-2069 (1989)), the reason why we used heparin-agarose column to purify it from the bacterial lysate (Roch, et al., J. Biol. Chem., 267:2214-2221 (1992)). In the course of purification of KB75 and KB75δ expressed in bacteria, we noticed that the binding affinity of KB75δ to heparin-agarose column was less than that of KB75 (Fig. 11). The lack of the heparin-binding capacity of biologically active, synthetic peptides corresponding to the deleted sequence in KB75δ (Fig. 11), however, excluded the active involvement of the binding to heparin or "heparin-like" molecules, which presumably present in the assay media or on the cell-surface, in the growth-promoting activity. Further evidence for the absence of contribution of heparin-binding capacity to the activity is that the inclusion of either heparin or heparin sulfate n the assay media (both at 100 μg/ml) failed to inhibit the effect of either 17-mer or KB75 (data not shown). A potential heparin-binding site on sAPP-695 predicted from the consensus sequence (Cardin, et al., Arteriosclerosis, 9:21-32 (1989)) is not on the active domain but on APP98-104 (Narindrasorasak, et al., J. Biol. Chem., 266:12878-12883 (1991)). Thus, although the sequence APP306-

335 may contribute to the heparin-binding capacity of sAPP-695 in its native form of the molecule, the growth-promoting activity of the domain in itself had little to do with the heparin-binding capacity.

In summary, using our bioassay on A-1 cells, we have demonstrated that the sequence APP305-335 contained the only active site of sAPP-695 and that the activity was represented by the 5 amino acid sequence RERMS (SEQ ID NO:1) (APP328-332).

The biochemical basis for the activity is still unknown and we suppose the presence of a cell-surface molecule(s) recognized by this domain of sAPP. Recent studies in our laboratory have revealed that the biological activity of this domain of sAPP is not confined to this particular cell line; the addition of synthetic peptides corresponding to this domain in the culture media caused significant morphological and biochemical changes in either neuroblastoma cells from rat brain or primary cortical neurons from newborn rate (manuscript in preparation). Thus, sAPP seems to be involved in the regulation of cellular homeostasis in various cell lines.

In a previous study, we found that cultured fibroblasts from the skin of AD patients produces lower amounts of APPs compared to fibroblasts from normal individuals (Ueda, et al., Ann. Neurol., 25:246-251 (1989)). There had been several reports describing the reduction of the relative proportion of APP-695 to the KPI-containing forms of APP in postmortem brains from AD patients (Tanaka, et al., Biochem. Biophys. Res. Commun., 157:472-479 (1988); Johnson, et al., Science, 248:854-857 (1990); Neve, et al., Neuron 1:669-677 (1988)). More recently, Van Nostrand, et al., Pro. Natl. Acad. Sci. U.S.A., 89:2551-2555 (1992), reported a significant reduction of sAPP in the cerebral spinal fluid of live AD patients. These findings raised a possibility that, in addition to C-terminal β/A4 portion of APP which is involved in amyloidogenesis, the secreted portion of APP is also involved in the pathogenesis of AD. We expect that the biologically

active peptides and their antagonists described above will be useful tools to study the possible involvement of the biological activity of sAPP-695 in various experimental model systems for the pathogenesis of AD.

B. Localization Studies of APP

We have conducted studies to attempt to pin down the manner in which APP localizes within the brain to garner information about how APP may act to promote neurite growth. In one aspect of the studies, we sought to determine the involvement of APP in aberrant sprouting in AD by analyzing control and AD sections from the frontal cortex double-immunolabeled with a monoclonal antibody against APP and a polyclonal antibody against GAP43. In another aspect of these studies, we examined cell adhesion in the context of APP localization.

It will be appreciated that secreted amyloid precursor protein has been shown to have trophic effect in cultured fibroblasts and to increase the survival and neuritic outgrowth of cortical neurons *in vitro*. Based on other studies, it has been suggested that APP might induce this trophic activity by regulating cell adhesion. Furthermore, APP 695 levels are unregulated during the process neurulation. Recent studies in the brains of Alzheimer's disease (AD) patients using antibodies against the growth associated protein GAP-43 have shown that a subpopulation of the abnormal neurites in the plaques are actually aberrant sprouting neurites. Furthermore, about 50% of these sprouting neurites co-express APP. Therefore, we expect that APP is involved in the processes of neuritic outgrowth and response to synaptic damage in AD.

EXAMPLE XIII

Role of APP in Aberrant Sprouting of Neurons

We examined the role of APP in the aberrant sprouting of neurons through conducting co-labelling studies on tissue sections of AD autopsy confirmed brain tissue. Sections were labelled with a monoclonal antibody specific for APP and a polyclonal antibody specific for growth associated proteins.

Thereafter, the sections were transferred to slides and imaged through laser morphography.

Tissue

Twelve autopsy cases from the Alzheimer's Disease Research Center at the University of California, San Diego, were utilized in the present analysis. Six of the cases had clinical histories of AD which was confirmed at autopsy. The average age of the AD cases was 79 ± 11 yr, with a postmortem delay of 5 ± 4 h. The other 6 cases were clinically and histopathologically free of neurological disease. The average age of these control cases was 78 ± 8 yr with a postmortem delay of 5 ± 3 h. In each of the 12 cases, blocks taken from the frontal cortex were fixed in 2% buffered paraformaldehyde for 72 h at 4°C. The tissue was serially sectioned at 40 μm with the Oxford vibratome.

Monoclonal Antibody

The mouse monoclonal anti-APP (Clone 22C11, Boehringer Mannheim, Indianapolis, IN) was used to label control and AD sections. The antibody was obtained by immunizing mice with purified recombinant APP fusion protein. The antibody was purified from ascites by ion-exchange chromatography and tested elsewhere. Additional sections were incubated with the 22C11 antibody adsorbed with purified recombinant APP (1.5 μg/ml) obtained from bacteria, as previously described. The rabbit polyclonal antibody against GAP43 was produced by immunizing the animals with a gel-purified protein as previously described. The antibody specificity and characteristics as to human brain tissue have been previously described.

Labeling

To assess the colocalization of GAP43 positive sprouting neurites and anti-APP immunoreactivity in the areas of synaptic pathology, 40 μm vibratome sections from control and AD frontal cortex were double-labeled with anti-APP and anti-GAP43 and secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas red, respectively. Optimal

and consistent results were obtained in vibratome sections washed for 25 min with 0.025% Triton X-100, followed by blocking with 20% normal goat serum and overnight incubation at 4°C with a mixture of the monoclonal antibody APP (10 μg/ml) and polyclonal antibody against GAP43 (1:250). Sections were the incubated in a mixture of FITC-conjugated horse anti-mouse IgG (1:100), followed by Avidin D Texas red (1:100; Vector Laboratories, Inc., Burlingame, CA).

Imaging and Analysis

The double-labeled sections were transferred to SuperFrost plus slides (Fisher Labs, Tustin, CA) and mounted under glass cover slips with anti-fading media containing 4% n-propyl gallate (Sigma Chemical Company, St. Louis, MO). The sections were studied with the Bio-Rad MRC-600 laser scanning microscope mounted on an Axiovert Zeiss microscope. This system permits the simultaneous analysis of double-immunolabeled samples in the same optical plane.

From each case 10 random fields within the frontal cortex were imaged. Images of 0.2 μm thick optical sections of the neuropil displaying the APP/GAP43 immunolabeled plaques were recorded. All series of sections from each case were digitized under standardized conditions maintaining the same gain, aperture and black levels, as previously described. We found that quenching of the fluorescent images was minimized by testing the effect of different periods of laser exposure on the tissue, using the condenser no. 2 and the use of anti-fading media. Each series of sections was scanned through a total of 10-15 μm and thus, each complete series was composed of approximately 50-75 dual optical sections. The digitized video images were processed and stored on 650 Mbyte rewriteable optical disk cartridges for subsequent display and analysis. An average of 10 fields per case were imaged with each field measuring 3,350 μm and containing an average of 4-5 neurons. The pixel intensity of anti-APP immunoreactivity was quantified with the Bio-Rad MRC software, as previously described.

Serial optical sections of the double-immunolabeled plaques were transferred via the Ethernet into the Silicon Graphics Iris 4D/210VGX. As previously described, visualization of the three-dimensional (3-D) relationship between the APP-positive dystrophic neurites and GAP43-immunostained aberrant sprouts was achieved with the aid of the ANALYZE software package. Using this software, 3-D volumetric images were rendered and viewed as colorized move loops on the Silicon Graphics Iris terminal.

Statistical analyses of the results were conducted using the STAT VIEW II (Abacus Concepts, Inc., CA) software package running on a Macintosh personal computer. Statistical comparisons between AD and the normal control group were done with the unpaired, two-tailed Student's *t*-test.

Laser confocal imaging of sections double-immunolabeled for APP and GAP43 showed that, in the control neuropil of the frontal cortex 2.0 ± 0.23% of the presynaptic buttons immunostained with anti-GAP43, contained anti-APP immunoreactivity. In contrast, in AD, 3.3 ± 0.4% of the presynaptic terminals that were immunolabeled with anti-GAP43 contained anti-APP immunoreactivity (Figure 13; $P < 0.05$). Most of these anti-APP-immunolabeled terminals were slightly larger than the average terminal immunolabeled with anti-GAP43. In AD, anti-APP displayed a stronger immunostaining intensity in the pyramidal neurons of the frontal cortex as compared to controls (Figure 13), while anti-GAP43 immunoreactivity was decreased in the cell body of the neurons, but increased in the neuritic processes. A prominent finding in the AD cases was the presence of intensely anti-GAP43 and anti-APP immunoreactive abnormal neurites in the plaques (Figure 14).

Computer-aided serial section analysis and 3-D reconstruction showed that anti-APP was colocalized with anti-GAP43 in 57.5 ± 11% of the aberrant sprouting neurites (Figures 14 and 15). Rotation of the 3-D images showed that anti-GAP43 immunolabeling was associated with both round and

fusiform abnormal neurites in the plaque, while anti-APP was mostly associated to the round distended profiles. Detailed laser confocal analysis of the pattern of distribution of GAP43 in the dystrophic neurites showed that it was present in most of the neurites in a granular fashion, while APP was present diffusely in low concentrations with a few foci of increased immunoreactivity (Fig. 14).

These results show colocalization of APP immunoreactivity in GAP43-immunoreactive sprouting neurites in the plaques of the AD neocortex as well as with a subpopulation of GAP43-positive synapses. Furthermore, other studies have colocalized APP with synaptophysin-immunoreactive terminals, suggesting that APP might reach the sprouting neurites by fast anterograde axonal transport. The other line of evidence that supports a role of APP in sprouting comes from developmental studies of APP expression during embryogenesis. These studies have shown that the levels of APP-695 increase 10-fold between E6.5 and E8.5, suggesting the possibility that APP is developmentally regulated during the embryogenesis of the neural tube. In normal mice, expression of APP and GAP43 mRNA increases throughout fetal and post-natal development declining in adults. In contrast, in trisomy 16 mice, APP and GAP43 message levels were elevated by twofold. The change in GAP43 compartmentalization observed in AD was accompanied by an increase in APP immunoreactivity in the neurons and synapses. These findings are consistent with previous studies where APP immunoreactivity was increased in the AD hippocampus.

The colocalization of APP and GAP43 in neuritic plaques could be linked to a probable role of these two proteins in cell surface adhesion, in fact, GAP43 might play a role in substratum-cell adhesion required in growth processes. In this framework, APP upregulation in AD appears to be associated with the aberrant sprouting response in the brain as a consequence of the synapse loss and neuro-degeneration. In this model of AD pathogenesis, β/A4 amyloid deposition is

the result of the upregulation and abnormal processing of APP, but not the cause of the neuropil damage in the first place. It is, however, possible that the chronic deposition of amyloid in the brain might contribute to some extent in accentuating or accelerating the neural damage, which is currently the prevailing thinking in the field based on the neurotoxic effect of β/A4-protein. Consistent with our hypothesis, recent immunohistochemical studies after excitotoxic damage in the rat hippocampus, have shown increased immunoreactivity of APP in the astrocytes in the lesioned site.

EXAMPLE XIV

Cell Adhesion Studies

To study the role of APP in the process of neuritic outgrowth further, we examined the distribution of APP and its relationship to the patterns of GAP-43 expression in the neonatal rat brain through cell adhesion studies.

Animal Tissues

The forebrains of eight 1-day-old (P1) Sprague-Dawley rats were obtained and fixed by immersion in 2% paraformaldehyde in phosphate buffered saline (pH 7.4) for 72 hrs at 4°C. The tissue was serially sectioned at 40 μm with the Dosaka microslicer.

Antibodies

The mouse monoclonal anti-APP (Clone 22C11, Boehringer-Mannheim, Indianapolis, IN) was used to label the neonatal rat brain sections. The antibody was obtained by immunizing mice with purified recombinant APP fusion protein. The antibody was purified from ascites fluid by ion-exchange chromatography and tested elsewhere. The polyclonal antibody against GAP-43 was produced by immunization of rabbits with a gel purified protein as described previously. The antibody specificity and characteristics have been described. Further double-immunohistochemical analyses were done with the rabbit polyclonal antibody that recognizes phosphorylated epitopes on medium and heavy subunits of neurofilament proteins in axons (Sigma Chemical Company, St. Louis, MO), with a rabbit

polyclonal antibody against glial fibrillary acidic protein (GFAP, Biogenex, San Ramon, CA) and with the mouse monoclonal antibody against synaptophysin (SY 38, Boehringer-Mannheim).

Double Immonolabeling and Laser Confocal Imaging of Neurites in the Neonatal Rat Brain

To assess the colocalization of GAP-43 positive growth cones and anti-APP immunoreactivity in the rat brain, 40 μm vibratome sections were double-labeled with anti-APP and anti-GAP43 and secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas red, respectively, as described previously. Briefly, sections were incubated overnight at 4°C with a mixture of the monoclonal anti-APP antibody (10 μg/ml) and polyclonal anti-GAP43 antibody (1:250). Sections were the incubated in a mixture of FITC-conjugated horse anti-louse IgG (1:70) and biotinylated goat anti-rabbit IgG (1:100), followed by Avidin D Texas red (1:100) (Vector Laboratories, Inc., Burlingame, CA). The double-labeled sections were transferred to SuperFrost plus slides (Fisher Labs., Tustin, CA) and mounted under glass cover slips with anti-facing media containing 4% n-propyl gallate (Sigma). Additional sections were double labeled with the following antibody combinations: (a) APP/neurofilament; (b) APP/GFAP; and (c) SY38/GAP43. The sections were studied with the Bio-Rad MRC-600 laser scanning microscope mounted on an Axiovert Zeiss microscope for simultaneous analysis of double-immunolabeled samples in the same optical plane.

From each neonatal rat brain, sections were imaged at level PO-26 according to Paxinos, et al., Atlas of the Developing Rat Brain. Images of 1 μm thick optical sections of the neocortical and hippocampal neuropil displaying the APP/GAP-43 immunolabeled neurites were recorded. All series of optical sections were digitized under standardized conditions maintaining the same gain, aperture and black levels, as described previously. Each series of sections was scanned through a total of 10-15 μm. The digitized video images were processed and stored on 650 Mbyte rewriteable

optical disk cartridges for subsequent display and analysis.

Laser confocal imaging of sections double-immunolabeled for APP and GAP-43 showed that, in the neuropil of the frontal parietal region, abundant neuritic processes contained strong anti-GAP-43 immunoreactivity (Figs. 16A and B). These processes were more abundant in the inner layers of the neocortex and extended to the intermediate zone, where lower fiber density was observed (Fig. 16B). The superficial layers of the neocortex displayed an intense GAP-43 immunoreactivity mainly localized in fibers oriented in parallel to the pial surface (Figs. 16A and B). In the inner layers, APP was localized to the cell bodies of the neurons that extended their growing processes toward the intermediate and superficial layers (Figs. 16A, B, C). However, in the intermediate and superficial layers of the neocortex, APP was co-localized with GAP-43 positive neuritic fibers (Figs. 16C, 17B, C). In the superficial layers APP was also occasionally co-localized with GAP-43 positive horizontal fibers (Fig. 17A). APP immunoreactive neuritic processes were ramified in their distal portion (Fig. 17B) and contained anti-neurofilament immunoreactivity (Fig. 17D), although they were anti-GAP negative. Anti-synaptophysin immunolabeled the occasional presynaptic terminals present in the neuropil. Only a few of these terminals contained GAP-43 immunoreactivity, most of GAP-43 immunoreactivity was concentrated in the neuritic processes adjacent to the presynaptic boutons (Fig. 17E).

In the hippocampus GAP-43 immunoreactivity was concentrated in the neuritic processes of different strata of the hippocampus, although the immunoreactivity was less abundant in the somata of pyramidal and granular cells (Fig. 18). APP immunoreactivity was present in some perikarya of pyramidal and granular cells and was colocalized with GAP-43 positive neuritic processes in the stratum pyramidale (Fig. 18). Furthermore, strong APP immunoreactivity was found in the molecular layer, while GAP-43 immunolabeled the stratum lacunosum (Fig. 18B).

The present study showed colocalization of APP immunoreactivity in GAP43-immunolabeled neurites in the neonatal rat neocortex and hippocampus. These neuritic processes corresponded to long fibers originating in neurons distributed in the inner layers. Previous studies in the neonatal rat brain have shown that these processes correspond to growth cones that eventually will establish the cortical microcircuits. Moreover, biochemical studies have shown that neonatal rat brain contains high levels of APP, identified in the Western blot with an anti-C terminal antibody as a band at 110 to 130 kD. The presence of APP in the neurites of the immature rat brain suggests that this molecule might be involved in the process of neuritic outgrowth in combination with other molecules, by at least three different mechanisms: (1) by acting as an indirect or direct trophic agent, (2) by functioning as an adhesion molecule, and (3) by modulating the protease/protease inhibitor activity.

In this regard, previous studies have shown that APP promotes neuronal and fibroblast growth, and a fragment corresponding to the β/A4 region enhances the survival and neurite extension of hippocampal neurons in vitro. Furthermore, APP mediates neuronal cell-cell and cell surface adhesion similar to N-CAM in sympathetic cells. APP is produced in a secretory and non-secretory form, in different cell lines. The human glioma Bu-17 cells produces only the non-secretory form of APP, and in these cells APP immunoreactivity is accumulated in the growth cones, suggesting that the non-released form of APP may play a role in process growth through regulating cell adhesion. In addition, the N-terminal region of the APP molecule, with KPI domain, has been identified as PN-II, a potent inhibitor of chymotrypsin, coagulation cascade proteases, and forms stable complexes with EGF binding proteins and the gamma subunit of NGF. Thus, the microenvironment produced by the balance between protease/protease inhibitor activity is probably a central mechanism that regulates neuritic outgrowth. However,

further analysis of the various isoforms of APP expressed during neuritic outgrowth is necessary in order to understand the mechanisms by which APP is involved in neuritic sprouting. In AD, APP is accumulated in abnormal neuritic processes in the plaques that are enriched with GAP-43. In this regard, APP upregulation or abnormal processing in AD might be associated with an abnormal growth response in the brain as a consequence of the ongoing synaptic pathology.

C. Use of APP Moieties Containing the RERMS Active Sequence in Treating Neurons

During the progression of Alzheimer's disease (AD), several cortical and subcortical regions of the CNS suffer neuronal and synaptic loss. Recent studies have shown that the AD brain is capable of mounting an aberrant sprouting response in those denervated areas. As discussed in the previous section, immunocytochemical analyses with an antibody against growth associated protein (GAP43) have shown that in AD there is an extensive aberrant sprouting response in the hippocampus and neocortex, mostly associated with plaque and synapse loss. Furthermore focal synapse loss in the neuropil is associated with β/A4-amyloid deposition in the neuritic plaques.

As will be appreciated, the ß-protein is derived from a larger precursor protein, APP. Antibodies against specific regions of the APP molecule immunolabel the plaque dystrophic neurites and the APP immunoreactivity of the hippocampal neurons is increased in AD. In this regard, previous studies have shown that APP mediates neuronal cell-cell and cell surface adhesion similar to neural cell adhesion molecule (N-CAM). APP also promotes fibroblast growth and a fragment corresponding to the A4 region enhances the survival and neurite extension of hippocampal neurons *in vitro*. In addition, the N terminal region of the APP molecule, with the Kunitz protease inhibitor (KPI) domain, has been identified as PN-II, a protease inhibitor probably involved in neuritic outgrowth.

Taken together these factors suggest that APP and its

fragments might serve as growth promoting factors and could be produced in AD in response to ongoing synaptic pathology. It has been reported that PN-2 participates in regulation of neurite extension (Van Nostrand, et al., Science, 248:745-748 (1989); Oltersdorf, et al., Nature, 341:144-147 (1989)), blood coagulation processes (Cole, et al., Biochem. Biophys. Res. Commun., 170, 288-295 (1990); Van Nostrand, et al., 1990; Smith, et al., Science, 248:745-748 (1990)) and in wound-healing process (Cunningham, et al., Brain Res. Rev., 16:95-96 (1991)). Little is known, however, about the physiological function of sAPP-695 which lacks KPI domain, in spite of the evidence indicating that APP-695 is the major form in the brain (Neve, et al., Neuron 1:669-677 (1988); Ponte, et al., Nature, 331:525-527 (1988); Tanaka, et al., Biochem. Biophys. Res. Commun., 157:472-479 (1988); Kōnig, et al., Mol. Brain Res., 9:259-262 (1991)).

It has also been shown in recent studies that there appears to be a delicate balance between PN-2 and APP. For example, it is indicated that neuronal death in AD might be associated with a relative decline in sAPP-695 as compared to PN-2. Johnson, et al., Science, 248:854-857 (1990); Tanaka, et al., Biochem. Biophys. Res. Commun., 157:472-479 (1988). Moreover, it has been shown that a relative increase of PN-2 in relation to sAPP-695 is manifested in transgenic animals as AD type pathology. Quon, et al., Nature, 352:239-241 (1991).

In addition, a relatively increased level of PN-2 to sAPP-695 has been observed in aged rats, Neve, et al., Neuron, 5:329-338 (1990), and behaviorally impaired old rats, Higgins, et al., Proc. Nat'l Acad. Sci. USA, 87:3032-3036 (1990). However, some conflicting data exists with regard to this observation. Golde, et al., Neuron, 4:253-267 (1990); Konig, et al., Mol. Brain Res., 9:259-262 (1991).

We have also shown that sAPP-695 is secreted from cells and has a growth promoting effect on fibroblasts. Saitoh, et al., Cell, 58:615-622 (1989). Consequently, in connection with our discovery that only a small portion of the amino acid sequence of sAPP-695 is responsible for the growth enhancement

in fibroblasts, we have also discovered that fragments of sAPP-695 containing the RERMS sequence, discussed above, exhibit a profound ability to enhance the growth of neurons.

Moreover, we have been able to employ a rat CNS neuroblastoma cell line that responds to APP by extending the neurites. This allows a relatively simple assay to identify peptides that participate in neuron growth promotion. While our primary assay employs this neuroblastoma cell line, we have also used primary neuronal culture successfully to demonstrate APP activity. Further, we have tested all of the peptides discussed above incorporating the RERMS sequence in connection with their ability to participate in neuron regeneration. As an additional aspect of the assay, we conduct a blind quantification of the neurite length of the cells using a computer assisted laser morphometric analysis as described in Examples XIII and XIV, above.

To study the biological function of secreted forms of APP (sAPP) on neurons, used a clonal CNS neuronal line B-103 that does not synthesize or secrete APP from Dr. Schubert, at the Salk Institute for Biological Studies. When B-103 cells were plated at low density in a synthetic serum free medium, neurite outgrowth was promoted by the conditioned medium from APP-695 or APP-751 over-producing cells and also by the bacteria produced sAPP-695 (named KB75) and sAPP-751 (named Nex II). A 17-mer peptide (APP 17-mer) corresponding to Ala-319 to Met-335 of APP-695 that is active in restoring the normal growth of A-1, the fibroblast cell line that secrets little APP and grows poorly (Saitoh, et al., supra. (1989)), also induced neurite outgrowth from B-103 at concentrations higher than 10 Nm.

Interestingly, we have shown that this effect does not occur through the enhancement of cell adhesion to substratum. For example, a binding assay using the 17-mer labeled with ^{125}I as a ligand demonstrated a specific, saturable binding with first-order kinetics. We predicted Kd to be 20 ± 5 Nm and Bmax 80 ± 8 fmole/10^6 cells. Indeed, this binding can be

displaced with KB75.

We have also demonstrated that the reverse sequence 17-mer, described in Example VII, neither induced neurite outgrowth nor competed for the binding. The bacteria produced sAPP fragment without the active 17-mer sequence (named KB75$_\Delta$) did not compete with ^{125}I-labeled 17-mer for binding and had significantly reduced neurite promoting effect as compared to KB75. As discussed above in connection our growth assay of A-1 cell line in accordance with Examples V and VI, we have shown nearly identical effect of the various peptides (17-mer, 11-mer, and 5-mer) on neurite growth as compared to growth promoting effect on the A-1 cell line. Indeed, the 11-mer induced the same extent of neurite extension as the 17-mer and fully replaced its binding. It was found that the sequence of 5-mer, RERMS (SEQ ID NO:1) (App328-332), within the APP 11-mer, was the shortest active peptide, although the concentration of peptide required for the neurotrophic activity was one order of magnitude higher than 17-mer.

Also parallel to our discussion above, in connection with Examples V and IX, the peptide sequence RMSQ (SEQ ID NO:2) (App330-333) was found to antagonize the neurotrophic effects of both KB75 and Nex II at higher concentrations.

Accordingly, we believe that sAPP induced neurite extension is due to a cell surface binding mechanism, and, that the 11-mer represents the active sequence for this function.

To conduct our binding assays discussed above, we iodinated our peptides in accordance with the following Example. As will be understood, for our study, only the 17-mer was iodinated, however, any of the peptides discussed herein could be suitably iodinated and assayed in accordance with the disclosure herein.

EXAMPLE XV

Iodination of the 17-mer

The 17-mer was iodinated with [^{125}I]Bolton-Hunter reagent (Amersham Co., 2000 Cl/mmol, 500 μCl) according to the manufacture's instructions. In brief, the peptide solution

(10 μg in 10 μl of 0.1 M $NaHCO_3$ buffer, pH 8.5) was incubated with the dried reagent at 4°C overnight. The reaction was quenched with glycine (100 μl of 1 mg/ml solution of H_2O) and the iodinated peptide was separated from the unincorporated reagent through Sephadx G15 column (1 cm^2 x 25 cm) saturated with PBS containing 0.15% gelatin. The specific activity of the obtained ^{125}I-17-mer was 40 to 60 Ci/mmol.

EXAMPLE XVI

Binding Assay

The cells in the late log phase were harvested in PBS, and spun down at 1,200 g for 5 min. Thereafter, the cells were suspended in DMEM/5%FCS. The suspension (400,000 cells/ml) was seeded in 24-well plate (0.5 ml/well) and the plate was kept at 37°C in a CO_2 incubator for three hours. The plate was then transferred on top of ice and the media was immediately replaced with ice-cold PBS containing 0.15% gelatin (PBSG). After 30 min, the binding reaction was started by replacing the PBSG with fresh, ice-cold PBSG containing ^{125}I-17-mer and various peptides as indicated above. The reaction volume was 0.25 ml.

After various lengths of time, the binding media was aspirated and the cell layer was washed three times with 0.5 ml of ice-cold PBSG. The radioactivity remaining on the cell layer was then recovered in 0.5 ml of the extraction buffer (0.5% Triton X-100, 0.1 M $NaHCO_3$ pH 7.4) and was counted by γ-counter. All the binding assays were conducted in triplicate.

Through the use of the above labeling studies, we were able to demonstrate that the binding of ^{125}I-17-mer to B-103 cell layer was time-dependent (Fig. 19) and saturable (Fig. 20) with Kd values of 20 ± 5 nM and Bmax values of 80 ± 8 fmole/10^6 cells (means ± SEM of three determination each done in triplicate). Further, the binding was sequence-specific because 1) the 17-mer, but not reverse sequence 17-mer, could displace the binding (Fig. 21) and 2) the C-terminal 11-mer of the 17-mer fully displaced the binding while N-terminal 8-mer did not (Fig. 21).

As well, the binding of the 17-mer was displaced by KB75 but not at all by KB75δ (Fig. 22), suggesting that the 17-mer represents at least one of the sequence(s) on the secreted form of APP-695 which is/are recognized by cell-surface molecule(s), i.e., a cell surface receptor. Moreover, when we conducted inhibition studies, wherein various fragments of 11-mer peptide (Fig. 23 and Table 1) were utilized, we observed that the 5 amino acid sequence RERMS (SEQ ID NO:1), in the mid-section of the 11-mer is essential for the binding activity. Such results are indicated in Table 1.

TABLE 1

Peptide	Sequence	IC_{50} (nM)	
KB75	APP20-591	11.7±2.3	
KB75δ	APP20-591 without 296-35		No inhibition at 1μM
17-mer	AKERLEAKHRERMSQVM (APP319-335)	12.5±4.4	
rs17-mer	MVQSMRERHKAELREKA		No inhibition at 1μM
8-mer	AKERLEAK		No inhibition at 1μM
11-mer	AKHRERMSQVM	35.9±16.5	
N5	AKHRE		48±2% inhibition at 1 mM
N4	KHRE		32±3% inhibition at 1mM
M6	HRERMS	765±167	
M5	RERMS	842±111	70±5% inhibition at 1 mM
C7	ERMSQVM		61±7% inhibition at 1 mM
CS	RMSQVM		

It will be appreciated that Table 1 provides a summary of the inhibition of the radiolabeled 17-mer in its binding to and with the B-103 cell layer by the indicated peptides. The

concentration of ^{125}I-17-mer was 10 nM. Nonspecific binding was defined as the binding in the presence of 1 μM unlabeled 17-mer. The figures presented were calculated from the data shown in Figures 3-5 (means ± SEM of at least three determination each done in triplicate).

It will also be appreciated that additional culture systems may be used. For example, found that PC12 mutant cell line ø141 is susceptible to a high concentration of APP whereas the parent PC12 is not, under the identical conditions. Thus, one may develop effective assays in accordance with the present invention through use of other cell lines that are known to those of skill in the art.

D. Neurotrophic Properties of the APP Moieties Containing the RERMS Sequence in *in vivo* Rabbit Models

Considerable evidence supports the hypothesis that APP is neurotrophic, at least under some circumstances. We have recently demonstrated, as was discussed in greater detail above, that the neurotrophic properties of APP in cell culture are fully preserved in a the 17-mer of the present invention. This 17-mer has been tested on a neuronal cell line (B-103) that does not express APP. Neuroblastoma cultures of B-103 treated with the 17-mer had more neurite-bearing cells, and longer neurites, than untreated cells. Other neurotropic factors such as NGF and FGF also have been shown to alleviate neuronal loss resulting from some types of injury. If APP is neurotrophic in vivo, then it would be anticipated that administration of the growth-promoting segment of APP might

attenuate the neuronal loss of behavioral deficits associated with neuronal injury, such as that accompanying CNS ischemia. In this experiment, we evaluated this 17-mer peptide in a rabbit model of focal CNS ischemia.

EXAMPLE XVII

Spinal Cord Ischemia Model

Male New Zealand white rabbits weighing between 2 and 3 kg were individually housed and allowed food and water ad lib for three to five days prior to surgery. Under halothane anaesthesia, a paramedial abdominal incision was used to expose the aorta at the level of the renal arteries. Small-diameter plastic tubing was placed around the aorta just distal to the left (more caudal) renal artery. The ends of the tubing were threaded through a small plastic button and then through a large-diameter plastic tubing, to form a snare ligature.

The incision was closed around the tube so that the free ends of the tubing were accessible externally. The rabbits were allowed to recover from the anesthesia for at least three hours and appeared to behave normally prior to ischemic insult. More detailed descriptions of this model have been published previously, as will be appreciated by those of skill in the art.

Prior research has demonstrated that this model produces stable, reproducible results and is sensitive to pharmacological manipulations that improve neurological outcome following ischemia.

EXAMPLE XVIII

Infusion of Rabbit Spine with the 17-mer

The 17-mer was dissolved in isotonic saline and diluted to concentrations of 200, 500, or 1000 nM. Control animals received saline. Saline or peptide was administered l.t. via spinal catheter using conventional techniques 20 min prior to ischemia and once daily for three days thereafter. Following random assignment, each animal received .2 ml of the 17-mer or saline, followed by a .1 ml saline flush.

EXAMPLE XIX

Producing an Ischemic Condition in the Rabbits

Spinal cord ischemia was produced by pulling and clamping the small tube around the aorta. Complete paraplegia and anesthesia were present in the hindquarters of all rabbits within two minutes of occlusion. At the end of the ischemic period, the tubing was released, restoring blood flow through the aorta. The tubing was removed, and the abdominal wall was closed with a suture, and the skin was closed with surgical clips. Depending on the duration of the ischemic insult selected, all grades of neurologic function were encompassed; from complete recovery to permanent total hindlimb paraplegia.

Postoperative care of the rabbits included manual expression maneuvers twice daily on paraplegic animals. Animals were maintained for three days following ischemic insult. Rabbits dying within this period were excluded to ensure that no aortic thrombosis were used in data analysis.

EXAMPLE XX

Monitoring Neurological Function

Neurologic function was evaluated by two observers blind to animal treatment. Evaluations were conducted at 24 hours and again on the third day following ischemia (experimental day 4). Animals were classified by the presence or absence of paraplegia. Paraplegic animals, for example, showed more response to obnoxious stimuli in the hind limbs and were also totally incontinent. Rabbits that were not paraplegic were either normal or had some motor function in the hind limbs, even if only barely detectable. Bowel and bladder function were variable. If it was difficult to ascertain whether an animal was totally paraplegic, it was classified as partially paraplegic. Following the last evaluation on day 4, animals were sacrificed with Euthanol (Schering-Plough, Kenilworth, NJ).

After sacrafice, the spinal cords of the animals were rapidly removed and replaced in fixative (10% phosphate-buffered formalin) for immunohistochemical analysis and

histological evaluation.

EXAMPLE XXI
Data Analysis

In order to quantitate the effects of treatment of our animals with the 17-mer, we utilized quantal dose-response analysis techniques used to evaluate neurologic damage as a function of ischemic insult that have been previously described. A computerized curve-fitting process was used to fit logistic (s-shaped) curves to the fraction of abnormal animals as a function of ischemia duration. The duration of the ischemia required to produce paraplegia in 50% of a group of animals (ET_{50}) was computed. Differences in ET_{50}s between control and treatment groups were evaluated using t tests (two-tailed).

EXAMPLE XXII
Neurologic Outcome

Through the above methodology, we have shown that the administration of the 500 nM concentration of the APP-derived 17-mer significantly improved neurological outcome three days after spinal ischemia. The ET_{50} in control animals (n = 10) at 4 days was 30.12 +/- 1.77 min (mean +/- s.e.). Administration of 200 nM 17-mer (n = 11) produced a non-significant trend toward increased tolerance of ischemia (ET_{50} = 48.84 +/- 18.47 min). In the 500 nM condition (n = 13), the ET_{50} increased significantly to 41.39 +/- 4.64 min, $p < .05$). Administration of 1000 nM 17-mer (n=8) was without effect (ET_{50} = 27.52 +/-5.12). These dates are summarized in Figure 24.

None of the other 17-mer concentrations significantly improved neurological status at the 24 hr evaluation, although the 500nM does produced a trend (p = .10) toward increased tolerance of ischemia. The ET_{50} values for the control 200, 500 and 1000 nM concentrations were, respectively, 30.27 + 1.45, 30.19 + .87, 41.94 + 8.29 and 31.86 + 7.69. Although the 17-mer's administration did not significantly increase the duration of ischemia required to produce paraplegia, the pattern of results was similar to that of the results observed at four days.

EXAMPLE XXIII
Effective Dosages

It is interesting to notice that, as the dose of the 17-mer was increased beyond the 500 nM concentrations, there was a decrease in the neurotrophic effect. However, as will be appreciated, these findings are entirely consistent with those of that are known in the art.

While the 200 nM does produced a trend toward improvement, however, the 1000 nM does was completely without effect. In the 200 nM group, the absolute value of the ET_{50} was considerably elevated above that of the control group, although the variance in this condition was also increased. We have consistently observed similar increases in variability when animals are treated with suboptimal doses of neuroprotective agents (unpublished observations).

Neurotoxin-induced subcortical lesions were associated with increased APP synthesis and turnover in the brain regions innervated. This response was rapid, persisted for several days, and was not a response to general stress but was specific to differentation. Although APP production rapidly increases in brain injury, the rate of turnover also increases. Whether this is part of an injury mechanism, a repair mechanism, or unrelated is not apparent. However, because the APP promoter contains the heat-sensitive element, we believe that the induction of APP synthesis is part of the protective mechanism of cells. As will be discussed below, we have recently demonstrated that APP peptides containing the RERMS sequence (SEQ ID NO:1) can support the survival of rat primary cortical neurosis and also can promote the neurite extension of primary culture neurons and neuroblastoma. The latter might manifest an activity to stimulate the sprouting of neurons and to increase the synaptic contact. In connection with our above results, these findings suggest that APP acts in a supportive fashion in injury, as the trophic APP fragment improved neurological outcome following ischemia by increasing the synaptic density but not by preventing the neuronal loss.

EXAMPLE XXIV
Histological Examination

We were also able to demonstrate that the 17-mer did not alter the degree of necrosis in the spinal tissue. In addition, the application of the 17-mer did not alter the levels of neuronal cell death. Rather, the 17-mer appears to have significantly reduced the tau immunoreactivity of neurons. Moreover, there was a large increase in the synaptic density of the neurons due to the administration of the 17-mer.

The reparative activity of 17-mer is entirely consistent with our immunohistochemical analysis of the ischemic tissue to which the 17-mer was administered. While the 17-mer is ineffective in reducing necrotic tissue or number of neuronal loss, the 17-mer enhanced the number of anti-tau positive neuronal population. This result clearly indicates that the 17-mer has an effect on the plastic reaction of remaining neurons. Indeed, the increased synaptic densities associated with neurons that were treated with the 17-mer is further confirming of this mechanism. Thus, the effect of the 17-mer does not appear to be the direct protection of neurons under ischemic stress. Rather, the 17-mer appears to lead to promote the cells remaining after the ischemic insult to sprout and extend neurites and expand in synaptic density, in order to compensate for the lost neurons.

We have, thus, developed a protocol to capitalize on the promotional aspects of the peptides of the present invnetion and certain drugs that have been shown to have a neuroprotective effect. We combine the advantages of the apparently neuropromotional abilities of the peptides based on the RERMS sequence (SEQ ID NO:1), and drugs that we are developing therefrom, with the advantages of certain neuroprotective drugs, by treating a patient, first with a neuroprotective drug, followed by the administration of a peptide or drug based on the RERMS sequence (SEQ ID NO:1). As will be understood, one may combine the two moieties in a single administration, or one may reverse the order. However,

in either event, the two activities of each moiety act to supplement the activity of the other. Essentially, it appears as though the neuroprotective drug acts to shield the neural cells from the action of ischemic agent or event, yielding a larger number of neurons that survive the ischemia. Further, the peptide, containing, or drug based upon, the RERMS sequence (SEQ ID NO:1) will aid in the promotion and thriving of those neurons that survived.

EXAMPLE XXV

Neuroprotective Drug Systems in Conjunction with Drugs Based on the RERMS Sequence (SEQ ID NO:1)

We have used the neuroprotective drug MK-801 to enhance the effectiveness of the treatment with the 17-mer or an analogue thereof. MK-801 is an n-methyl-deaspartic acid glutamate antagonist. Thus, as will be appreciated by one of skill in the art, a variety of glutamate antagonists are suitable for use in the present invention. As well, protein kinase antagonists and calcium channel blockers have shown similar effect. Moreover, agents such as trifluoperazine (Stelazine®, Smith, Kline & French) and chlorpromazine (Thorazine®, Smith, Kline & French) have been shown to be effective. We also expect that the calpain inhibitors should be effective, such as leupeptin and E-64. In addition, other neuroprotective drugs will be readily known to those of skill in the art or can be readily found without undue experimentation.

Upon administration of these drugs to a model, such as the rabbit ischemic model, discussed in connection with Example XVIII, we observe a marked increase in neural cells surviving after 24 hours, as compared to the number of neural cells surviving when the rabbits are treated with the 17-mer or an analogue thereof alone. However, after 48 hours, or, more so after 4 days, there are substantially larger numbers of neural cells thriving with enhanced synaptic densities.

In addition to the ischemic model above, we have also tested *in vitro* hypoxia and hypoglycemia models, and have found that similar results are observed as in the ischemia model

above. As will be appreciated by one of skill in the art, these findings are relevant to Alzheimer's disease because, the recent epidemiological study demonstrates that myocardial infarct is a risk factor for this disease. Additionally, we have conducted preliminary tests on a model to study high oxygen pressure and H_2O_2 effects, since it is suggested that radicals generated at the time of reperfusion after ischemic insults might be responsible for neurodegeneration observed after ischemia. Also, radicals are major players in the aging process. Thus, we will evaluate these insults using different cell lines listed above

E. Memory Enhancement and Increase of Synaptic Density in Rat Models

We have discovered that APP moieties and analogues based on the 5-mer RERMS sequence (SEQ ID NO:1), and in particular the 17-mer of the present invention can induce both morphological and behavioral changes when injected in rat brains.

The general protocol that we employed in arriving at this dicosvery was the direct infusion of the 17-mer, a peptide having the reverse sequence of 17-mer, or artificial cerebrospinal fluid (CSF), as a control, were infused into 20 month-old rat brain ventricles using a mini osmotic pump. The animals were tested for memory performance using the Morris Water Maze paradigm (MWM). After the last behavioral test, the animals were sacrificed and their brain morphologically examined.

We found that the administration of the 17-mer resulted in an increase in synaptic densities in hippocampus, frontal cortex, and temporal cortex. The increased synaptic density was reflected at the behavioral level by an enhancement of the memory retention. These results suggest that APP-695 is involved in the memory.

EXAMPLE XXVI

Morris Water Maze Animal Behavioral Protocol

We used a total of 47 aged (21 months) and 9 young (4 months) female Fischer rats in our experiment to determine the

effect of the peptides of the present invention on memory and brain tissue morphology. Spatial learning and memory were tested in a water maze (Morris, J. Neurosci. Methods, 11:47-60 (1984)). The maze consisted of tank 152 cm in diameter, painted black on the inside and filled with tap water (room temperature) to a depth of 34 cm. The invisible platform, supported by a base resting on the bottom of the pool, was a 5x5 cm Plexiglas black square placed 3 cm under the surface of the water. The visible platform was an elevated platform with a 6-cm-high white base, clearly visible for the rats from the surface of the water.

The pool was located in the center of a room with numerous extra maze cues (e.g. shelves, tables, the computer). During testing, the ceiling lights were turned off and indirect illumination was provided by 2 lights located on either side of the pool and directed one toward the ceiling, and the other toward the floor. These lights provided diffuse illumination throughout the room so that the extra maze cues were visible from the pool, but did not illuminate the inside of the pool, which was dark, obscuring the location of the black platform. For descriptive data collection, the pool was divided into 4 equal quadrants formed by imaginary lines intersecting at the center of the pool, at right angles.

Such lines in turn intersect the edge of the pool at the arbitrary cardinal start locations called North, South, East, and West. The platform was placed at the center of the South-West (SW quadrant or the North-East (NE) quadrant. The swim path of the rats, path distance, path length in each quadrant, and escape latency (time to reach the platform) were monitored using a video camera, and videotrack images were digitized and analyzed using a computer program designed for water maze data analysis (San Diego Instruments, San Diego, CA).

At the start of a trial, a rat was placed at one of the 4 cardinal start locations, facing the wall of the pool. As the rat was released in the water, a pneumatic switch was pressed to start the timing and data collection by the video camera and computer system. If the rat did not locate the

platform with 90 seconds, it was manually placed on the platform for 15 seconds before the next trial. If the rat did locate the platform within 90 seconds, the trial was ended by pressing the pneumatic switch, and the rat was allowed to stay on the platform for 15 seconds before the next trial.

Each rat was given a block of 4 trials every test day. The start locations were ordered in a semirandom manner. The first and last trial of each day were started from one of the two locations farthest from the platform, but the start location of the first trial was never the same on two consecutive days. The start location of the second and third trials was a random choice between one of the two remaining possibilities.

The data was analyzed using the program "Statistica" (Stat Soft, Tustin, Oklahoma).

EXAMPLE XXVII

Peptide and Vehicle Iinfusion

The rats had cannulae implanted into the right lateral ventricle. The cannulae were connected to Alza model 2002 osmotic minipump (Alza Corporation, Palo Alto, CA) placed subcutaneously in the dorsal region of the neck/back. Animals were infused either with artificial CSF (saline solution), with the 17-mer peptide at 1mM, or with the peptide of reverse-sequence 71-rem at 1mM. Both peptides were dissolved in artificial CSF as vehicle. The infusion was carried out throughout the experiment, at a rate of 0.25 μl/hr.

EXAMPLE XXVIII

Animal Behavior Testing

We used the Morris Water Maze protocol described in Example XXVI as follows.

Training:

The rats were first trained for 5 days to escape from water onto a platform in the water maze with the visible platform. To avoid the possibility that the rats use memory cue to reach the platform, its location was changed randomly every day. At the end of the visible training, the time to reach the platform was not different between the old and young

groups (data not shown) suggesting that there is no physical, visual, or motivational impairment in the old rats.

The rats were then trained for 5 days with the invisible platform which was located at the center of the SW quadrant. The time that the rats required to find the platform did not change significantly during the last 3 days of testing. As an index of the learning performance, we used the mean of the times for animals to locate the invisible platform of the last 3 days. This mean was significantly higher for the old rats than for the young rates (17.7 s - 8.8 +/ vs 8.5 +/- s, $p<0.003$).

Therefore, the group of old rats showed reduced learning and memory ability when compared to the group of young rats. It should be noted that these differences in time could not be attributed to reduced swimming capacities in the old group, because they performed as well as the young rats in the test with the visible platform.

We grouped old rats into 3 groups for infusion with either 17-mer, artificial CSF, or the reverse 17-mer. The variability of the behavioral performance was greater in the old group than in the young group. Therefore, the old group contained some animals which performed as well as the young ones, and some that performed poorly. The animals were ranked from best to worst for their score of the last 3 days of testing (time to reach the platform) in the invisible test before operation.

The group assignment was in the order of A-B-C in the triplet containing the best 3 rats. For the next triplet, the assignment order was C-B-A. We then alternated this assignment order for the following triplets until all the rats were assigned to three groups. Thus, the mean scores of behavior for three groups are similar. The mean score of group A was 17.2 seconds +/- 5.3; the mean score of group B was 19.2 seconds +/- 11.9; and the mean score of group C was 18.0 seconds +/- 8.6.

Infusion:

The old rats were then implanted with minipumps

containing either peptides 17-mer at 1 mM, dissolved in artificial CSF, reverse 17-mer, dissolved in artificial CSF, or artificial CSF alone as the control. The group A received the peptide 17-mer, the group B received the artificial CSF alone, and the group C received the reverse 17-mer. This information was not known by the experimenter who performed the post-operation behavioral tests.

Infusion Testing:

One week after the operation, the rats were re-trained in the water maze first with a visible platform and then with an invisible platform located in the center of the SW quadrant (old location). For the test, two weeks after the operation, the invisible platform was moved to the center of the NE quadrant (new location). The animals were scored for their path length in the SW quadrant, where the platform used to be present.

In this test, we mainly test the retention of the memory (memory for the old location), although this test includes the aspect of memory acquisition (learning the new location). We considered the score as the mean value of the last 3 days of testing, because they did not change during that period.

The score of the group A was 249 +/- 133; the score of the group B was 214 +/- 135; and the score of the group C was 258 +/- 185. The differences between these scores of the 3 groups did not reach statistical significance (p values > 0.1360). However, our analysis (ANOVA) had only compared the average path length of each group (mean score of the last 3 days of each test). Thus, because each group contained rats that ranged from "normal" to "very impaired," an effect of the peptide on a subpopulation of these rats (e.g. only on the normal rats, or only on the impaired rats) would not have been detected by analyzing the means of the groups. Therefore, we decided to carry out correlation analysis.

The correlation analysis between the performance before the peptide infusion and the path length in the SW quadrant (while the platform was in the NE quadrant) after the 17-mer infusion revealed that there was an extremely significant

negative correlation (R=-0.59, p<0.001). No significant correlation was found for either group B or C (p values > 0). Thus, among the old animals that had received the 17-mer peptide, those that had a normal learning capacity stayed more in the SW quadrant where a hidden platform used to be than those that had a memory impairment.

Finally, we compared the effect of the 17-mer in impaired and non-impaired animals regarding the path length in the SW quadrant with the platform in the NE quadrant. The old rats were considered non-impaired if their score before operation was within the range of 3 standard deviations of the score of the young rats; they were considered impaired if their score before operation was over 6 standard deviations above the score of the young rats. The score and standard deviation of the young rats were 8.5 s +/- 1.8. Therefore, all the old rats with a score before operation under 13.9 s were considered non-impaired, and all the old rats with a score before operation above 19.3 s were considered as impaired. We found that, within the group of non-impaired animals, the path length in the SW quadrant (while the platform was NE) was significantly higher for the animals that had received the 17-mer than for those that had received artificial CSF or reverse 17-per (p value < 0.03). However, within the group of impaired animals, the path length in the SW quadrant was not significantly different between the animals that had received the 17-mer and those of the control group (p value > 0.58). Thus, the effect of 17-mer on the memory retention was observed on animals with a normal learning capacity but not on behaviorally impaired animals.

It seems that the infusion of the 17-mer peptide into animals fixated the memory of platform location. Thus, it is likely that these animals have difficulty learning a new location. Accordingly, we performed a correlation analysis between the times to reach the platform (mean value of the last 3 days) before infusion of peptide and the time to locate the new platform location after infusion. When we analyzed the scores before infusion with the score for new location

after infusion, we found that a significant negative correlation ($R=-0.56$, $p<0.03$) for the group A that had received the 17-mer peptide. No significant correlation was found for either group B or C (p values > 0).

Therefore, among the animals that received the 17-mer peptide, those that had a good score before operation took a longer time to find the platform at the new location than those that had a bad score before operation. To confirm this result, we compared the effect of the 17-mer in impaired and non-impaired animals. We found that, within the group of non-impaired animals, the mean score after the operation and infusion was significantly higher for the animals that had received the 17-mer than for those that received either the artificial CSF or reverse 17-mer. See Figure 25. Taken together as a control group those that received the artificial CSF and those that received the reverse 17-mer showed p values < 0.01.

However, within the group of impaired animals, the mean score after operation (platform NE) was not significantly different between the animals that had received the 17-mer and those of the control group (p value > 0.57). Therefore, our experiment detected a specific effect of the 17-mer on the non-impaired animals. This effect was detected as a poor performance in the water maze task to learn the new location. In other words, non-impaired animals that had received the 17-mer had difficulty in learning the new location of the platform as compared to non-impaired animals injected with control peptide or vehicle. Thus, it seems that the 17-mer peptide enhanced the retention of the memory so that animals go back to the old position resulting in the failure to go to the new location.

EXAMPLE XXIX

Morphological Studies

We also examine the rats brains after sacrafice. These studies are conducted almost identically to the rabbit histological studies discussed in connection with Examples __ and __. We observe similar results.

F. Design of Synthetic, Non-Peptide Drugs to Mimic APP Moieties' Containing the RERMS Sequence to Stimulate Growth of Neurons

As discussed above, APP is a neurotrophic factor. Thus, we believe that APP is required for the function and survival of neurons. Furthermore, because trophic factors are found to be effective in diminishing the damage of neurons caused by various insults, and because APP seems to be directly involved in the case of Alzheimer's disease, APP or APP-like moieties that contain the RERMS sequence should be prime candidates for the treatment of AD and other diseases or events in which neurons are impaired. For this purpose, we are developing non-peptidic APP-mimicking drugs to be administered systemically.

To model and design such non-peptidic drugs, we have devised a protocol that utilizes amino acid sequences of APP, and active fragments of APP, and nuclear magnetic resonance spectroscopy (NMR), or other spectroscopic techniques, to provide data that allows one to determine secondary and tertiary structures of proteins or peptides. Compounds fitting the model derived from the above information are then synthesized and tested in the biological assays, as described above, in order to determine their activity.

In general we model our compounds through subjecting the compound of interest to NMR, X-ray crystallography, circular dichroism, small angle neutron scattering (SANS) and other chemical procedures that offer further insight into the structure of the enzyme. In the preferred embodiment, NMR is employed to determine the overall molecular configuration and conformation.

In general, the NMR spectroscopy is conducted on a fragment of the APP moiety that contains the RERMS sequence. For example, each of the 5-mer, 11-mer, 17-mer, and 40-mer may advantageously be studied. Obtaining spectral data for one or more than one fragment allows graphic depictions of natural solution conformational changes as the surrounding amino acids are stacked around the RERMS sequence. However, it will be

appreciated that 17-mer alone is an ideal candidate for the molecular studies, since, it contains all of the activity of the 40-mer and enhanced activity over APP, yet is small enough in size to permit reliable and efficient analysis. When analyzing the 17-mer, Glucose 6-Phosphate Isomerase may advantageously be used as a model, because it has an overall homology with the 17-mer and X-ray crystallographic analyses have been conducted upon it so that accurate conformational data is available. A composite of the structure of the 17-mer as derived from its NMR spectrum as compared with NMR and X-ray data from Glucose 6-phosphate isomerase may be used. From these structures, an energetically stable conformation can be determined by one of skill in the art using routine molecular dynamic calculations.

Thereafter, the active domain of the APP moiety containing the RERMS sequence may be further identified through point mutation of the amino acid residues surrounding the active RERMS sequence. For example in the 17-mer, such point mutations are conducted upon the 9th through the 14th amino acid residues. Such point mutations allow us to determine the specific residues where substitution creates a variable activity from the native form. Each of the mutant forms are then analyzed with NMR to further assess the conformational changes produced thereby.

Once the point mutation studies have been completed and conformational change data that occurs due to such mutations are derived, one can readily calculate electrostatic potentials within the active domain. Electronic distribution within the active domain may be calculated by MNDO, AMI, or PM3 methods. Further, electronic distributions are also calculated for the point mutated peptides. From this combined data, the electrostatic potential required for activity within the active domain may be easily derived.

From the above data, relative configurations of molecules required for the activity can be determined. This step enables the design of a low molecular weight compounds that mimic the electrostatic properties and conformational aspects

required for activity. For example, the configurational requirements derived through the point mutation studies in conjunction with native sequence will indicate the required moieties and their configuration required for maximum activity. An appropriate hydrocarbon, or substituted carbon, skeleton can be fashioned to attain the configuration.

After fashioning the appropriate skeleton, the active moieties are substituted with synthetic molecules that possess homologous electrical and stearic properties to the original active moieties. Then, through calculating the structure and distribution of electrons of the compound obtained according to the above procedures one may identify the best fit. Compounds corresponding to the fit may be synthesized through routine synthetic chemistry.

NMR Spectroscopy

It is possible to obtain highly refined data about protein conformation through NMR spectroscopy. However, generally, there is an upper limit of the size of the peptide or protein for which the structure may be effectively determined with NMR. As will be understood, NMR operates upon the principle that all nuclei with odd mass numbers (i.e., ^{1}H, ^{13}C, ^{15}N, ^{17}O, ^{19}F, and ^{31}P), as well as those that have an even mass number, but an odd atomic number, have magnetic properties. At the same time, however, ^{2}H, ^{12}C, and ^{16}O are nonmagnetic.

Typically, NMR involves subjecting the molecule of interest to a magnetic field of a variable intensity at a fixed radio frequency. The output is expressed in terms of hertz (cycles per second). Further, the output provides basically four separate measurements: intensity (which in proton magnetic resonance (PMR) corresponds to the number of equivalent protons), chemical shift (the difference in frequency between the excitation of the moiety as referenced to a standard), width (provides an indication of the mobility of peptide), and coupling constants (measures interactions between nearby magnetic nuclei). Such measurements are referenced to a standard, such as tetramethylsilane or Tier's

salt.

Thus, when conducting NMR on a protein or peptide, one is able to see the affects or interactions of each of the functional moieties on the molecule. Since, in our analyses, we know the sequences of the proteins or peptide units, it is relatively simple to determine the directions that the functional units take with respect to one another, and the conformational bending that the overall chain of the molecule takes. Moreover, we are able to perform analyses for several peptide chain lengths that contain the RERMS active sequence. For example, we have analyzed NMR spectra from the 5-mer itself, the 17-mer, and the 40-mer. With each, conformational variations are shown by changes perceived for functional groups in conserved regions between the peptides.

Additionally, the NMR spectra of the point mutated 17-mer provide additional information about the molecular conformation and dynamics caused by such point mutations. Consequently, we are able to develop highly refined molecular conformation information from our NMR spectroscopy. A detailed discussion of the consideration and calculations that are involved in NMR determination of protein and peptide structure is provided in Wüthrich, Science, 243:45-50 (1989). In addition, the use of restrained molecular dynamics (RMD) for determining tertiary structural information of families of proteins from data regarding a single member of the family may be readily used by one of skill in the art for generating data about other peptides of proteins of the family. See Fujiyoshi-Yoneda, et al., Protein Engineering, 4:443-450 (1991).

In addition to NMR, circular dichroism, small angle neutron scattering (SANS) and other chemical procedures offer further insight into the structure of peptides or proteins. Of course, X-ray crystallography provides a comprehensive three dimensional structure that can confirm and integrate these other techniques. However, in contrast to X-ray crystallography, NMR is generally carried out in solution, which eliminates the perpetual problem of solving often

complex crystallization problems. Particularly, this is true in the case of relatively small peptides, where crystallization is virtually impossible. This is especially true in the case of our analyses of the conformational structure of the 5-mer and the 17-mer.

Interactive Model

In the use of any of these analytical constructs to discern the conformation and configuration of molecules and to design drugs therefrom to mimic the interacting molecules, it is important to appropriately develop an interactive model of the active peptide/receptor site. Previously, it has been impossible to determine which region on the APP protein growth promotion activity was located. Nor was it clear how to accurately predict the general construct of the receptor. However, with our discovery of the active region on the sAPP-695 molecule of the RERMS (SEQ ID NO:1) sequence as the locus of growth promoting activity, we may now study both active site geometry and molecular interaction. In addition we are also able to determine how overall conformation changes in regions extending both n- and c-terminally in the sequence, since, we are investigating several strata of sizes surrounding the active site of the protein.

Indeed, it is important to consider interactions beyond the active site. It will be noticed, for example, as discussed in connection with Example __ above, that while the RERMS sequence appears indispensable to the growth promoting activity of APP, the RERMS sequence itself contains only 10% of overall activity. We believe that these interactions assist in binding and thereby contribute to binding specificity. Thus, we have discovered that it is these interactions in conjunction with information obtained from the active site that make the design of specific promoter molecules a possibility. Moreover, this information additionally permits the design of specific promoter molecules for related but nonidentical molecules.

Many proteins within a cell have evolved from common progenitors. These proteins often share common activities,

for example, the APP family of proteins. Moreover, in the APP family, there is broad overlap in the APP molecules found in man and other animals. Exactly the same sequence as 40-mer is found in mammalian APPs (rat, mouse, and monkey). In the Drosophila "APP-like" molecule, there is a sequence REKVT (SEQ ID NO:18) (aa 423-427; Rosen, et al. 1989); the polarity of the side chains of the corresponding residues is the same as RERMS (K and R have basic, V and M have nonpolar, and T and S have uncharged polar side chains, respectively). The uniqueness and the high evolutionary conservation together suggest the fundamental role of the sequence in the physiological function(s) of sAPP. Accordingly, since the active sites of the proteins appear to be broadly conserved, it appears likely that the functions of the proteins are also preserved. Therefore to a large extent rational drug design relies on the identification of the familial similarities and hence drugs are designed to react broadly within a given family or class.

While all members of a class may provide a similar activity, such as growth promotion or regulation, each member may have only one specific target. Thus, successful rational drug design based on group similarities would provide molecules that also interact broadly. Where there are many members of a class acting within a restricted locale such as a single cell, a broadly acting drug would interact with any number of receptors on the cell from the group the receptors that may be responsible for particular actions.

The particular receptor for APP in its neuronal growth promotion role has not yet been isolated. It seems possible, however, that there is more than one receptor within neurons that may be activated through interaction with APP. Accordingly, it is advantageous to develop molecules of extreme selectivity to allow characterization and differentiation of the various roles that may attend APP. Without such selectivity, the interaction would be general and not specific. We believe that the present invention advantageously is capable of providing promoters with highly

specific interactions for a given member of a receptor class.

Previous methods for rational drug design require the crystallization of the target molecule of interest, followed by X-ray crystallography. However, the production of useful crystals is both difficult and time consuming. It first depends on the ability of the target molecule to be isolated and purified in sufficient quantity for crystallization. A large number of crystallization conditions often need to be tested and once a crystal is made that is of sufficient quality, additional crystals often need to be produced in order to have enough material for analysis. Further, not all molecules are readily purified or readily crystallized.

With our identification of the RERMS sequence as the active site for growth promotion in APP, and our construction of peptides homologous to the APP molecule surrounding the RERMS sequence, we believe that we can perform highly selective modeling. For example, through comparative studies of solution conformations of the 5-mer, 11-mer, 17-mer, and 40-mer, or any combinations, we expect to see tremendous details about the conformational changes exhibited by APP necessary for its growth promoting effects in neurons. Additionally, we need not use X-ray crystallography; we are able to utilize NMR techniques that avoid the problems of crystal formation that have attended prior techniques. However, it will be appreciated that X-ray crystallography may be utilized, as discussed above.

Obtaining Three-Dimensional Structure Data

In order to obtain data on the conformation of the active site when bound to the receptor or receptors, a variety of techniques can be used. These techniques include, circular dichroism, small angle neutron scattering, diffraction methods, including any combination of multiple and single isomorphous replacement, single or multiwavelength anomalous scattering methods, molecular replacement methods maximum entropy phasing, solvent-flattening methods and so-called "direct" methods used primarily to solve small-molecule structures. NMR allows one to accurately predict three-

dimensional attributes in the interaction. As well, X-ray crystallography may be used in order to generate specific coordinates for each of the non-hydrogen atoms in the complex. Coordinates for the hydrogen atoms can additionally be obtained using neutrons.

X-ray Crystallography

It will be appreciated by one of skill in the art that X-ray crystallography can be advantageously utilized to determine the molecular conformations exhibited by APP to promote neuron growth. Indeed, we anticipate the use of X-ray crystallography to confirm our conformational studies. For example, we expect that we will turn to larger molecular studies to further identify folding patterns that may either assist or hinder APP in its ability to engage the receptor or receptors. Moreover, X-ray crystallography will be used in efforts to co-crystalize the receptor or receptors in conjunction with any of the active peptides of APP, as they are determined.

It will be understood that X-ray crystallography permits three dimensional molecular analysis of a protein at the atomic level. Analysis requires the production of crystals and crystal production requires a pure concentrated product. Further, complexes of a protein of interest together with a second interacting molecule provides information on the conformational changes occurring within a protein in response to that second molecule. X-ray crystallography of a protein with its substrate, an antibody or a drug can provide information for rational drug design.

An X-ray diffraction pattern taken from a crystal looks like an array of spots of varying intensities. Each spot is related to one of the Fourier coefficients of the electron density pattern in the crystal. Thus, the electron density in the crystal can be reconstructed if a sufficient number of diffraction spots can be measured and the relative phase angles of the Fourier coefficients can be determined. Thus, a crystallized protein used in the practice of certain aspects of the present invention should be of sufficient quality to

obtain these measurements. For example, the spots of varying intensity in the diffraction pattern decay over time. It is quite difficult to work with diffraction patterns with half lives of less than 10 hours. However, it is possible to work with diffraction patterns having half lives as short as about 15 minutes to 3 hours, depending on the amount of structural data desired to be obtained.

Further, it is believed possible to work with crystals of even shorter half lives using equipment and computer programs more advanced than commonly available today. Additionally, not all crystals are of equal quality and poor crystals have large Bragg spacing diffraction limits. Thus, a workable crystal should have a Bragg spacing diffraction limit of less than 4 Å.

Determination of phase angles uses isomorphic replacement to insert atoms into defined positions in the crystal for diffraction data measurement. These angles provide information that permit the production of an electron density map. The map is then used to build an atomic model from which three-dimensional coordinates are measured that define the structure of the crystallized molecule.

The ability to design promoter molecules that act on a given receptor using information obtained by X-ray crystallography is dependent on the formation of crystals of purified enzyme. Methods for crystal production vary greatly and one cannot predict how readily a given molecule or complex will crystalize. However, those skilled in the art will recognize that a variety of methods for crystallizing can be attempted for any given peptide, protein, or protein/receptor combination, and that successful crystallization can be expected for a variety of those. Rational drug design additionally requires information about the interaction of a known receptor in order to accurately predict a potential receptor's effects on the active site of the promotor. Thus, crystals of the protein/receptor combination may be used to gather information on the conformation of the promotor in its inhibited bound form.

A three-dimensional structure can be obtained from the electron density data using a computer program such as TOM/FRODO. Further, a computer program, such as X-PLOR, can be used to improve the accuracy of the initial three-dimensional structure. There are a variety of computer programs available for analyzing X-ray crystallographic data. Those of ordinary skill in the art will recognize that many other such computer programs providing similar functions could also be used. From this data, the points of contact are identified both within the active site and the surrounding region. Invariant amino acids and consensus recognition sequences are identified. The data is further analyzed against available chemical data such as NMR, CD, SANS data and other data resulting from chemical procedures. This chemical data can provide additional information for the structural model.

The coordinates of the amino acids residing in and around the active site that may be gleaned from conducting X-ray crystallography on the various fragments containing the RERMS sequence provide a template that may be useful in exploring ways to enhance interaction with receptors.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: SUBSTANCES HAVING THE GROWTH-PROMOTING EFFECT OF AMYLOID PRECURSOR PROTEIN

(iii) NUMBER OF SEQUENCES: 18

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: UC035.001A

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Arg Glu Arg Met Ser
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg Met Ser Gln
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACAGTGTCA CTCGAGAGAG AATGGGAAGA GGCAGAA
37

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACTGAGTC CTCGAGCTAG ATCTCCTCCG TCTTGATATT
40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp Glu Asn
1 5 10 15

Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala Lys His
20 25 30

Arg Glu Arg Met Ser Gln Val Met
35 40

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Lys Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val
1 5 10 15

Met

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Lys Glu Arg Leu Glu Ala Lys
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Lys His Arg Glu Arg Met Ser Gln Val Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Lys His Arg Glu
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys His Arg Glu
1

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Arg Glu Arg Met Ser
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Glu Arg Met
1

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Arg Met Ser Gln Val Met
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Met Ser Gln Val Met
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Val Gln Ser Met Arg Glu Arg His Lys Ala Glu Leu Arg Glu Lys
1 5 10 15

Ala

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Arg Glu Lys Val Thr
1 5

WHAT I CLAIM IS:

1. An isolated peptide smaller than a native amyloid precursor protein (APP) that retains at least some neuronal growth promoting effect of APP, comprising at least five consecutive amino acid residues with side-chain polarities corresponding to the side-chain polarities of the sequence RERMS (SEQ ID NO:1).

2. A peptide according to Claim 1, wherein said peptide includes the sequence REKVT (SEQ ID NO:18).

3. A peptide according to Claim 1, wherein said peptide includes the sequence RERMS (SEQ ID NO:1).

4. A peptide according to Claim 1, wherein said peptide comprises 316 amino acid residues or less.

5. A peptide according to Claim 4, wherein said peptide comprises 150 amino acid residues or less.

6. A peptide according to Claim 5, wherein said peptide comprises 40 amino acid residues or less.

7. A peptide according to Claim 6, wherein said peptide comprises a 40-mer corresponding to residues 296-335 of APP.

8. A peptide according to Claim 6, wherein said peptide comprises seventeen amino acid residues or less.

9. A peptide according to Claim 8, wherein said peptide comprises a 17-mer corresponding to residues 319-335 of APP.

10. A peptide according to Claim 8, wherein said peptide comprises five amino acid residues or less.

11. A peptide according to Claim 10, wherein said peptide comprises a 5-mer corresponding to residues 328-332 of APP.

12. A peptide according to Claim 10, wherein said peptide comprises a 5-mer with side-chain polarities corresponding to the side-chain polarities of the sequence RERMS (SEQ ID NO:1).

13. A non-peptide drug effective in promoting neuronal growth, comprising a plurality of functional groups, said functional groups having a polarity, electron distribution and bond length corresponding to the polarity, electron distribution and bond lengths of the side-chains in the

peptide RERMS (SEQ ID NO:1).

14. The non-peptide drug of Claim 13, wherein said drug is produced by a method comprising:

generating structural data on a peptide that has neuronal growth-promoting activity and contains an amino acid sequence having a RERMS (SEQ ID NO:1) sequence or a RERMS-like sequence that has side-chain polarities corresponding to the side-chain polarities of the sequence RERMS, said peptide also containing a plurality of additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence located therein;

determining energetically stable conformations of said peptide by comparing said structural data to structural data of a model protein or peptide using molecular dynamics calculations, wherein said model peptide protein has substantial homology to SEQ ID NO:8;

determining the active domain in said peptide;

calculating the electrostatic potential of the active domain;

designing a low molecular weight non-peptide compound that has a structure that has an energetically stable conformation and matches the electrostatic potential, as determined in the foregoing steps;

synthesizing said non-peptide compound.

15. A drug comprising the non-peptide drug of Claim 13 or the peptide of Claim 1, wherein said drug is capable of crossing the blood-brain barrier of the central nervous system of an animal.

16. The peptide or non-peptide drug of Claim 15, wherein said peptide or non-peptide drug has a hydrophobic moiety attached to a terminal position of said drug in a manner such that said hydrophobic moiety will not interfere with the activity of said drug.

17. A method of promoting the regeneration of damaged neurons *in vivo* in a mammal, comprising:

identifying a mammal having damaged neurons; and

administering the peptide of Claim 1, the non-peptide drug of Claim 13 or the drug of Claim 15 to said mammal.

18. The method of Claim 17, wherein said damaged neurons are in the central nervous system (CNS) of said mammal.

19. A method of treating a condition associated with cerebral deposition of the amyloid β-protein in a human patient, comprising:

identifying a patient having said condition; and

administering the peptide of Claim 1, the non-peptide drug of Claim 13 or the drug of Claim 15 to said mammal.

20. The method of Claim 19, wherein said condition is Alzheimer's Disease (AD).

21. The method of Claim 20, wherein the patient has not yet developed complete Alzheimer's Disease symptoms.

22. A method of treating a neurological condition characterized by damage to neurons, comprising:

identifying a patient having said neurological condition; and

administering the drug of Claim 15 to said mammal.

23. The method of Claim 22, wherein said condition is selected from the group consisting of ischemic neurological damage, hypoxic neurological damage, denervation following injury or trauma to the CNS and glutamate toxicity.

24. The method of Claim 22, wherein the administering step additionally comprises administering a neuroprotectant drug to said patient.

25. The method of Claim 24, wherein the neuroprotectant drug is selected from the group consisting of MK 801, other glutamate antagonists, an inhibitor of calpain, chlorpromazine and trifluoperazine.

26. The peptide of any one of Claims 1-12 or non-peptide of either Claim 13 or Claim 14 for use as an agent to promote neuronal regeneration of neurons of the central nervous system

in vivo in an animal.

27. The peptide or non-peptide of Claim 26, for use in increasing the memory-retention ability of a mammal.

28. The peptide of Claim 26 for use as an agent to promote neuronal regeneration of central nervous system neurons in patients who have been subject to an ischemic or hypoxic event.

29. A method of increasing the memory-retention ability of a mammal, comprising administering a peptide of any one of Claims 1-12 or non-peptide of either Claim 13 or Claim 14 to said mammal in a manner such that said peptide or non-peptide enters the central nervous system of said mammal.

30. The method of Claim 29, wherein the peptide or non-peptide is administered by a method selected from the group consisting of intramuscular injection, intravenous injection, intrathecal injection, direct infusion into the central nervous system and oral administration.

31. A growth assay for testing the neuronal growth-promoting activity of a chemical substance, comprising:

adding a measured quantity of a said substance to a culture of a fibroblast cell line that is deficient in its secretion of APP, said cell line having a substantially slower growth rate than a normal fibroblast cell line not having said deficiency;

measuring the growth rate of the culture containing said substance;

measuring the growth rate of the culture in the absence of said substance; and

comparing the growth rate of the culture containing said substance with the growth rate of the culture in the absence of said substance, wherein an increase in the growth rate of the culture containing said substance relative to the growth rate of the culture in the absence of said substance indicates that said substance has neuronal growth-promoting activity.

32. The growth assay of Claim 31, wherein said

fibroblast cell line cell line A-1 derived from parent cell line AG2804.

33. The growth assay of Claim 31, wherein said fibroblast cell line is cell line B103.

34. A method of designing non-peptide drugs for use as neuronal growth promotion agents, comprising the steps of:

generating structural data on a peptide that has neuronal growth-promoting activity and contains an amino acid sequence having a RERMS (SEQ ID NO:1) sequence or a RERMS-like sequence that has side-chain polarities corresponding to the side-chain polarities of the sequence RERMS, said peptide also containing a plurality of additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence located therein;

determining energetically stable conformations of said peptide by comparing said structural data to structural data of a model protein or peptide using molecular dynamics calculations, wherein said model peptide protein has substantial homology to SEQ ID NO:8;

determining the active domain in said peptide;

calculating the electrostatic potential of the active domain;

designing a low molecular weight non-peptide compound that has a structure that has an energetically stable conformation and matches the electrostatic potential, as determined in the foregoing steps;

synthesizing said non-peptide compound.

35. The method of Claim 34, wherein the step of determining the active domain, comprises producing variants of said peptide having an amino acid substitution in the RERMS or RERMS-like sequence thereof.

36. The method of Claim 34, wherein the determining step comprises producing variants of said peptide having an amino acid substitution in said additional amino acids from the sequence of human amyloid precursor protein surrounding the RERMS sequence.

37. The method of either Claim 35 or Claim 36, wherein the determining step additionally comprises determining which of said variants provide neuronal growth-promoting activity.

38. The method of Claim 34, wherein said structural data of said peptide is generated by a method selected from the group consisting of nuclear magnetic resonance (NMR), X-ray crystallography, circular dichroism, and small angle neutron scattering (SANS).

39. The method of Claim 34, wherein the generating step comprises use of NMR.

40. The method of Claim 34, wherein the comparing step comprises use of glucose-6-phosphate isomerase as the model compound.

41. The method of Claim 34, wherein in the step of determining the active domain, active and inactive substitution parameters are derived from a growth assay, comprising:

adding a measured quantity of a said substance to a culture of a fibroblast cell line that is deficient in its secretion of APP, said cell line having a substantially slower growth rate than a normal fibroblast cell line not having said deficiency;

measuring the growth rate of the culture containing said substance;

measuring the growth rate of the culture in the absence of said substance; and

comparing the growth rate of the culture containing said substance with the growth rate of the culture in the absence of said substance, wherein an increase in the growth rate of the culture containing said substance relative to the growth rate of the culture in the absence of said substance indicates that said substance has neuronal growth-promoting activity.

42. The method of Claim 34, wherein the calculating step comprises use of a calculation protocol selected from the group consisting of: MNDO, AMI and PM3.

43. The method of Claim 34, wherein the designing step

comprises the steps of:

obtaining relative configurations of the RERMS or RERMS-like sequence and maintaining said relative configuration in order to design a hydrocarbon or substituted hydrocarbon skeleton that will maintain such relative configuration of amino acids;

substituting homologous model moieties onto said skeleton for the original amino acids to produce a model molecule;

calculating the structure and distribution of electrons on the model molecule to produce a structural and distributional result;

comparing the structural and distributional result with that for a known molecule that has been previously modeled;

repeating the substituting, calculating and comparing steps for a plurality of homologous model moieties;

determining the optimum homologous moiety for replacement of said homologous model moieties; and

synthesizing the molecule that comprises said optimum homologous moieties substituted onto said skeleton.

44. The method of Claim 34, wherein said non-peptide drug is also made capable of crossing the blood-brain barrier (BBB) in the central nervous system of an animal.

45. The method of Claim ?, wherein the step of making the drug capable of crossing the BBB comprises addition of a hydrophobic moiety to a terminal position of said drug in a manner such that said hydrophobic moiety will not interfere with the activity of said drug.

46. A pharmaceutical composition for the treatment of a medical condition associated with neuronal degeneration in the central nervous system of a mammal, comprising a peptide according to Claim 1, a non-peptide drug according to Claim 13 or a drug according to Claim 15 in an amount effective to

promote the growth or regeneration of neurons *in vivo* together with a pharmaceutically acceptable carrier, filler, or excipient.

47. The pharmaceutical composition of Claim 45, wherein said drug is present in said composition in an amount effective to treat a condition selected from the group consisting of a condition associated with cerebral deposition of amyloid β-protein, a hypoxic condition of neurons in the central nervous system, an ischemic condition of neurons in the central nervous system and a condition affecting memory retention.

48. The composition of Claim 45, wherein said composition additionally comprises a neuroprotectant drug.

49. The method of Claim 47, wherein the neuroprotectant drug is selected from the group consisting of MK 801, other glutamate antagonists, an inhibitor of calpain, chlorpromazine and trifluoperazine.

50. A pharmaceutical composition for increasing the memory-retaining ability of a mammal, comprising a peptide according to Claim 1, a non-peptide drug according to Claim 13 or a drug according to Claim 15 in an amount effective to increase the memory-retaining abilities of said mammal, together with a pharmaceutically acceptable carrier, filler, or excipient.

51. An antagonist of the neuronal growth-promoting activity of amyloid precursor protein.

52. An antagonist according to Claim 50, wherein the antagonist comprises a peptide containing the sequence RMSQ (SEQ ID NO:2).

Peptide	Sequence	
KB75	APP20-591, Kang sequence	
KB75δ	APP20-591 without 306-335	
40-mer	TPDAVDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVM (APP296-335)	SEQ ID NO: 5
14-mer	TPDAVDKYLETPGD	SEQ ID NO: 6
13-mer	GDENEHAHFQKAK	SEQ ID NO: 7
17-mer	AKERLEAKHRERMSQVM	SEQ ID NO: 8
reverse sequence17-mer	*MVQSMRERHKAELREKA*	SEQ ID NO: 17
8-mer	AKERLEAK	SEQ ID NO: 9
11-mer	AKHRERMSQVM	SEQ ID NO: 10
N5	AKHRE	SEQ ID NO: 11
N4	KHRE	SEQ ID NO: 12
M6	HRERMS	SEQ ID NO: 13
M5	RERMS	SEQ ID NO: 1
M4	RERM	SEQ ID NO: 14
M3	RER	
C7	ERMSQVM	SEQ ID NO: 15
C6	RMSQVM	SEQ ID NO: 16
C4	RMSQ	SEQ ID NO: 2

Figure 1

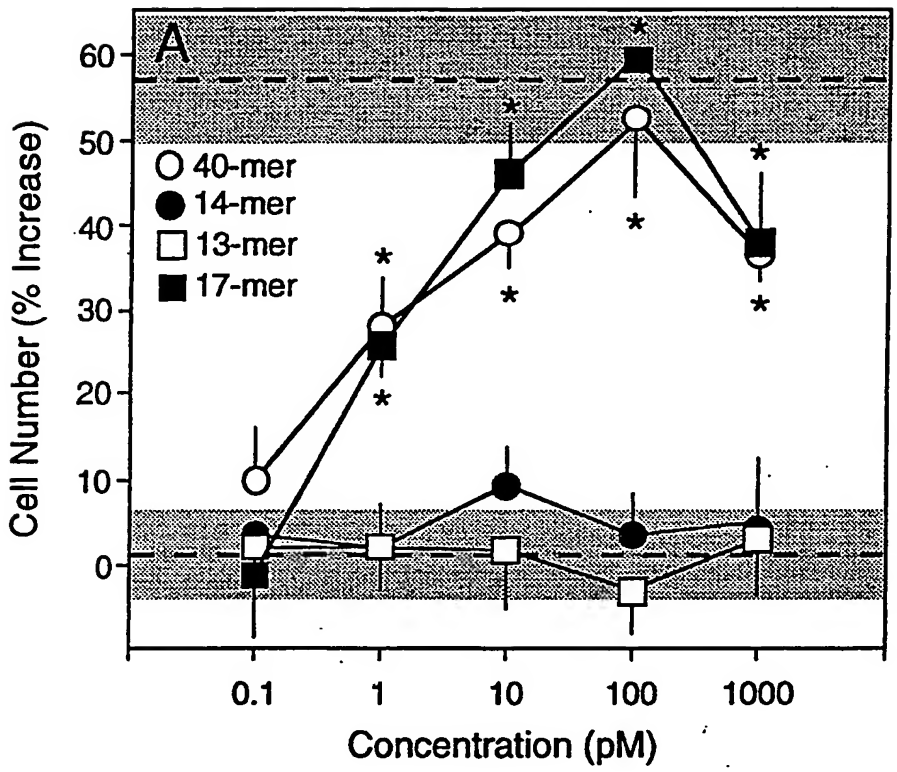
A
60
50
40
30
20
10
0
Cell Number (% Increase)
40-mer
14-mer
13-mer
17-mer
0.1
1
10
100
1000
Concentration (pM)

Figure 2

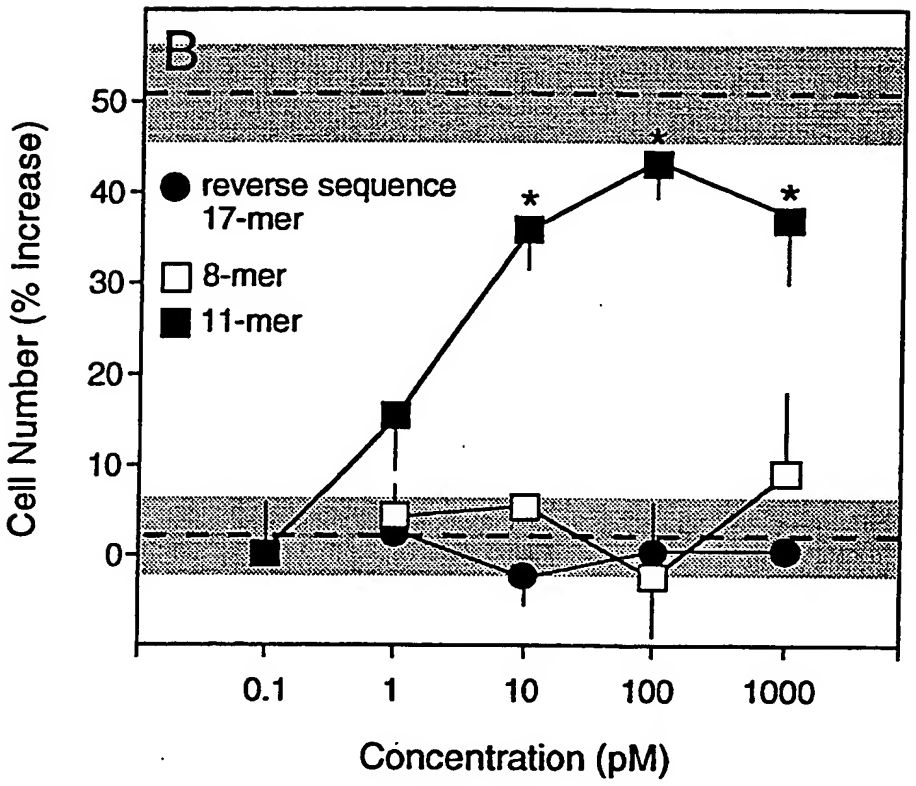
B
50
40
30
20
10
0
Cell Number (% Increase)
reverse sequence 17-mer
8-mer
11-mer
*
*
*
0.1
1
10
100
1000
Concentration (pM)

Figure 3

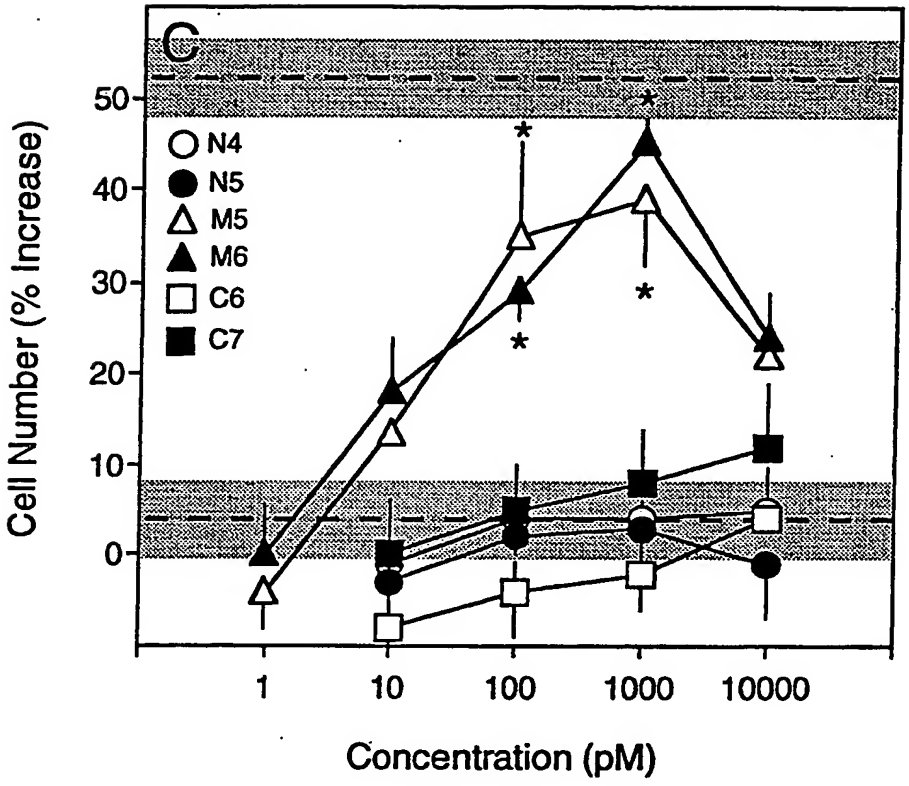
C
N4
N5
M5
M6
C6
C7
Cell Number (% Increase)
50
40
30
20
10
0
1
10
100
1000
10000
Concentration (pM)

Figure 4

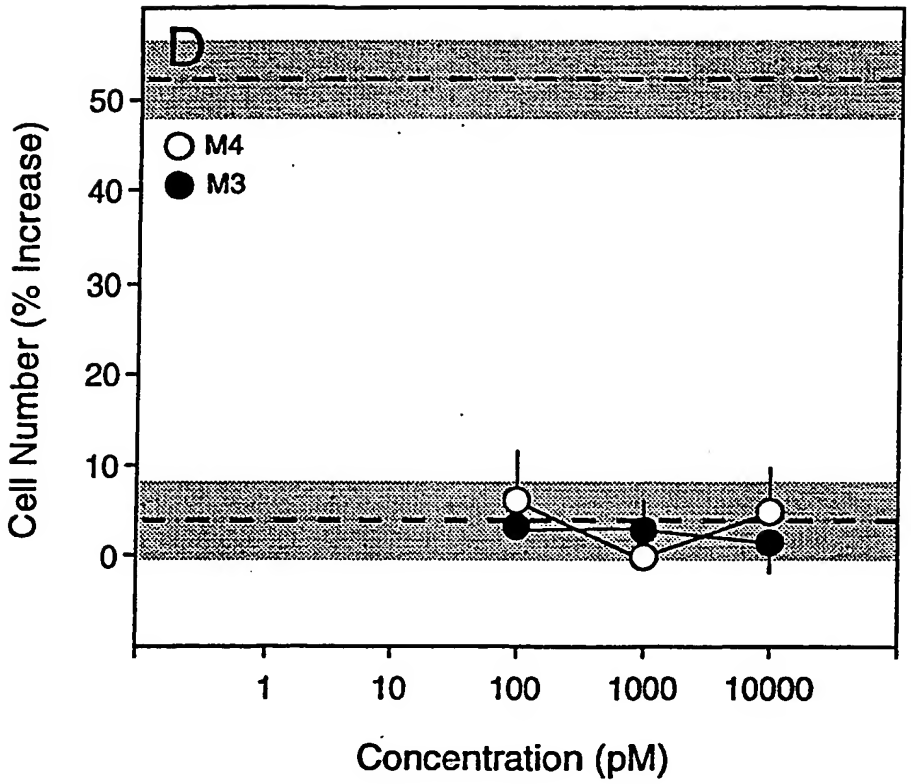
D
M4
M3
Cell Number (% Increase)
50
40
30
20
10
0
1
10
100
1000
10000
Concentration (pM)

Figure 5

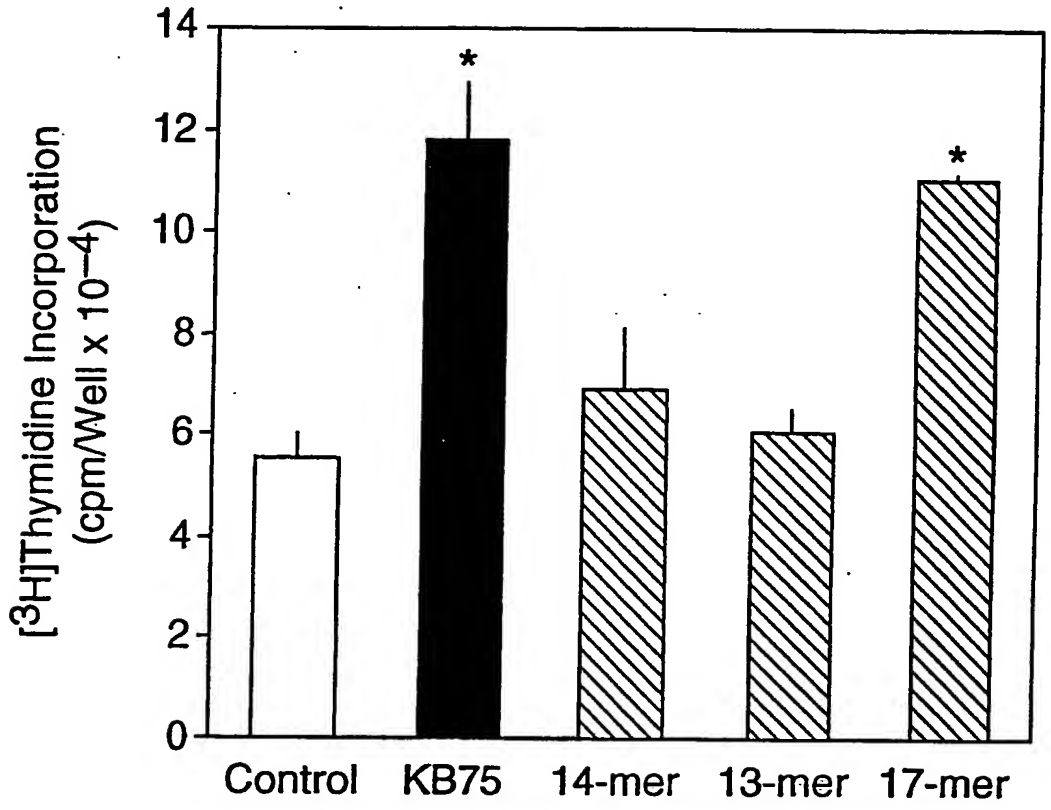
14
12
10
8
6
4
2
0
[3H]Thymidine Incorporation
(cpm/Well x 10−4)
*
*
Control
KB75
14-mer
13-mer
17-mer

Figure 6

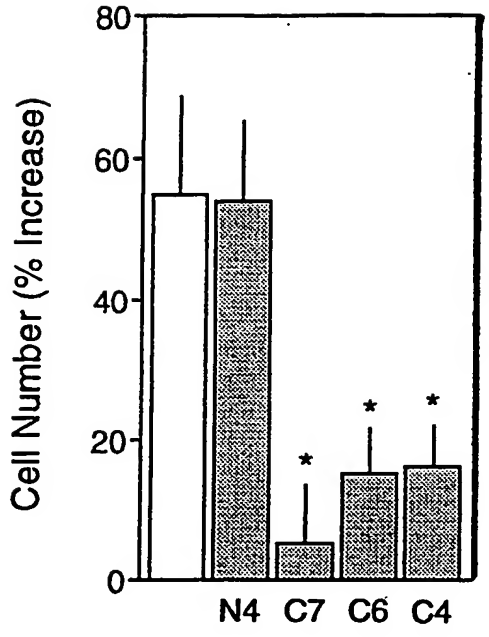

Figure 7A

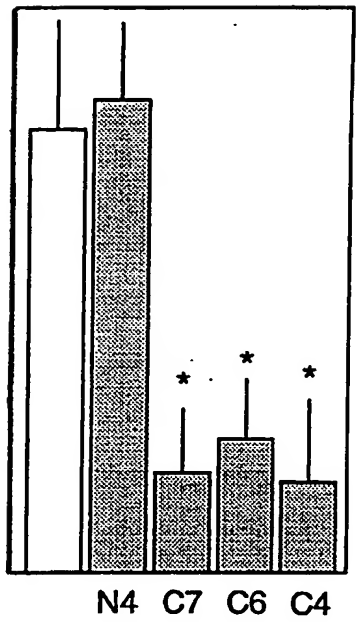

Figure 7B

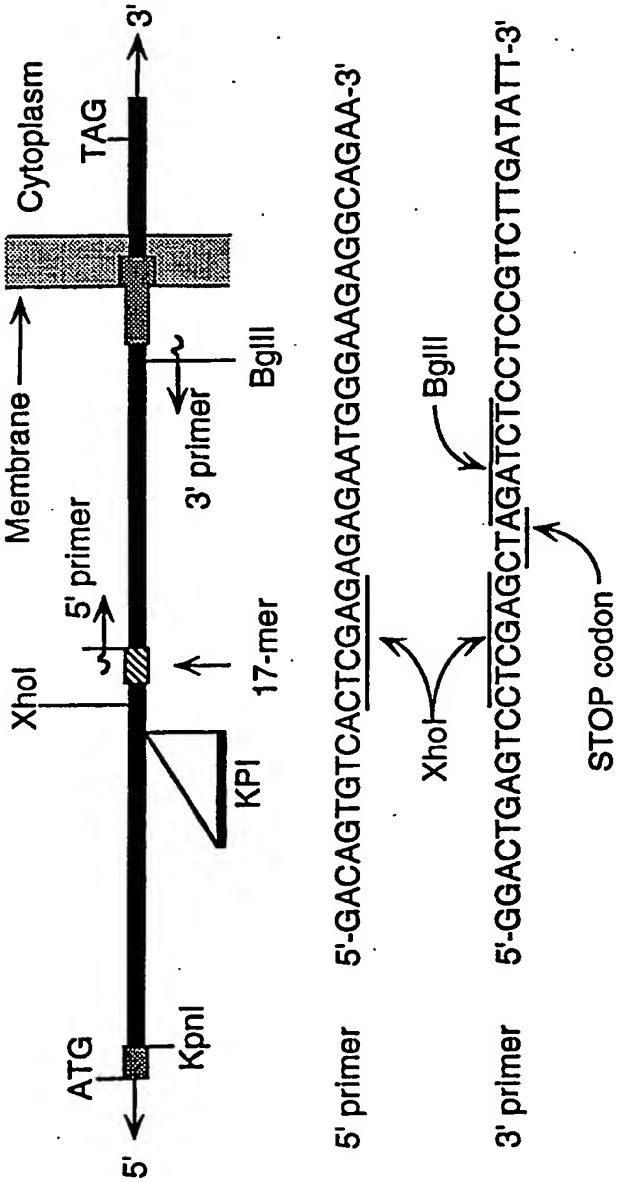
3'
Cytoplasm
TAG
Membrane
BglII
3' primer
5' primer
17-mer
XhoI
KPI
ATG
KpnI
5'
5' primer 5'-GACAGTGTCACTCGAGAGAGAATGGGAAGAGGCAGAA-3'
XhoI
BglII
3' primer 5'-GGACTGAGTCCTCGAGCTAGATCTCCTCCGTCTTGATATT-3'
STOP codon

Figure 8

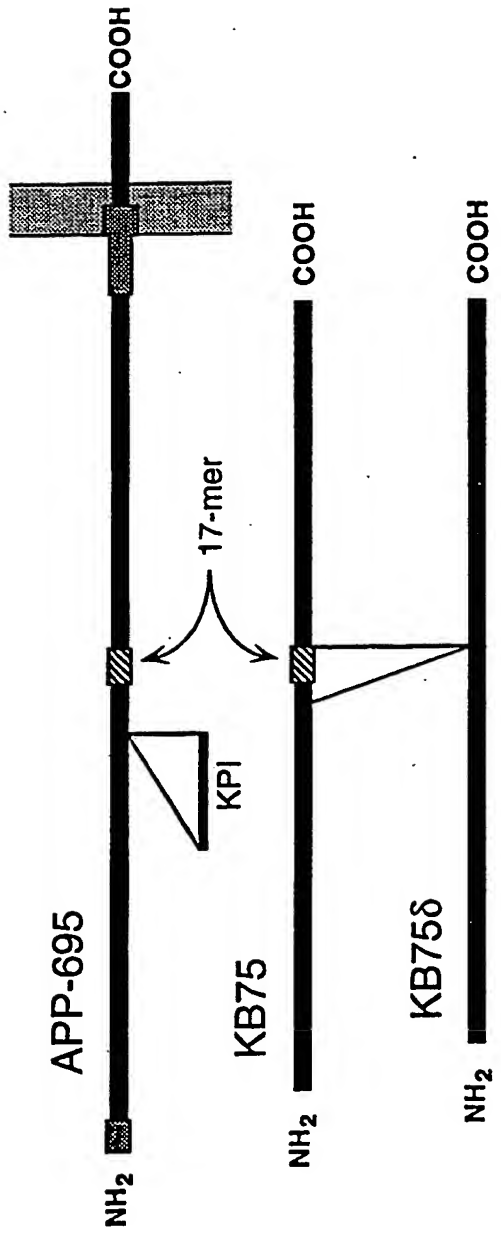

Figure 9

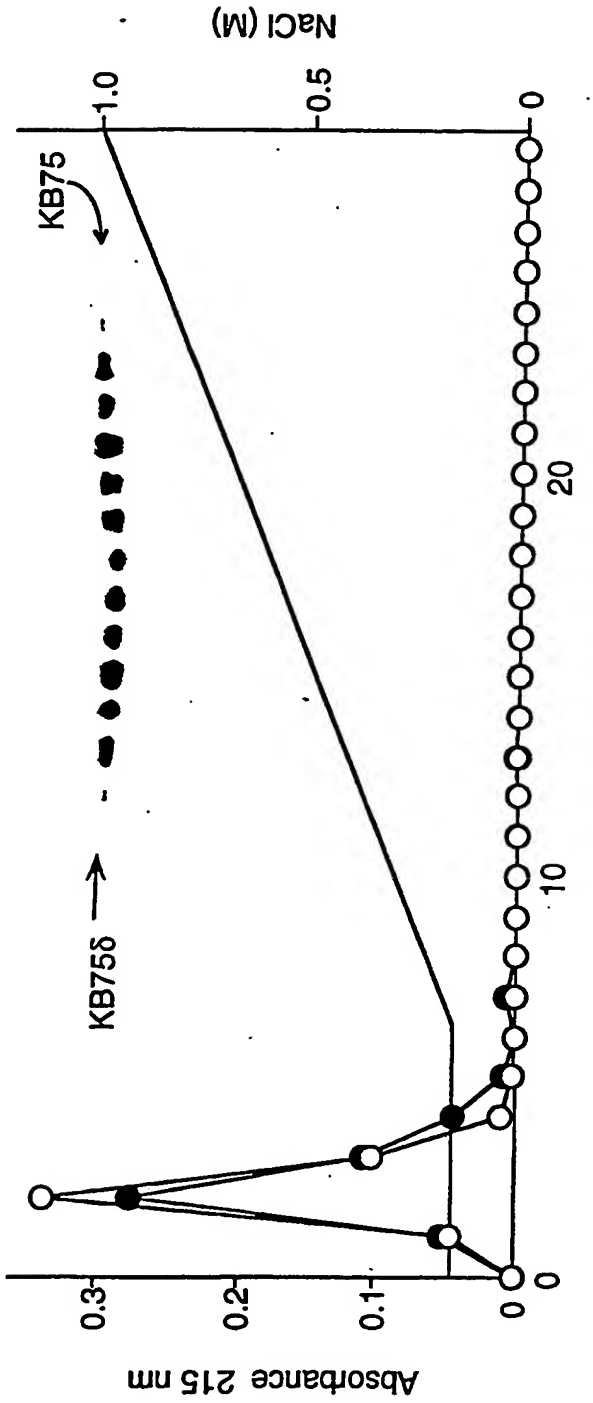
NaCl (M)
1.0
0.5
0
KB75
KB75δ
0.3
0.2
0.1
0
10
20
Absorbance 215 nm
Fraction Number

Figure 10

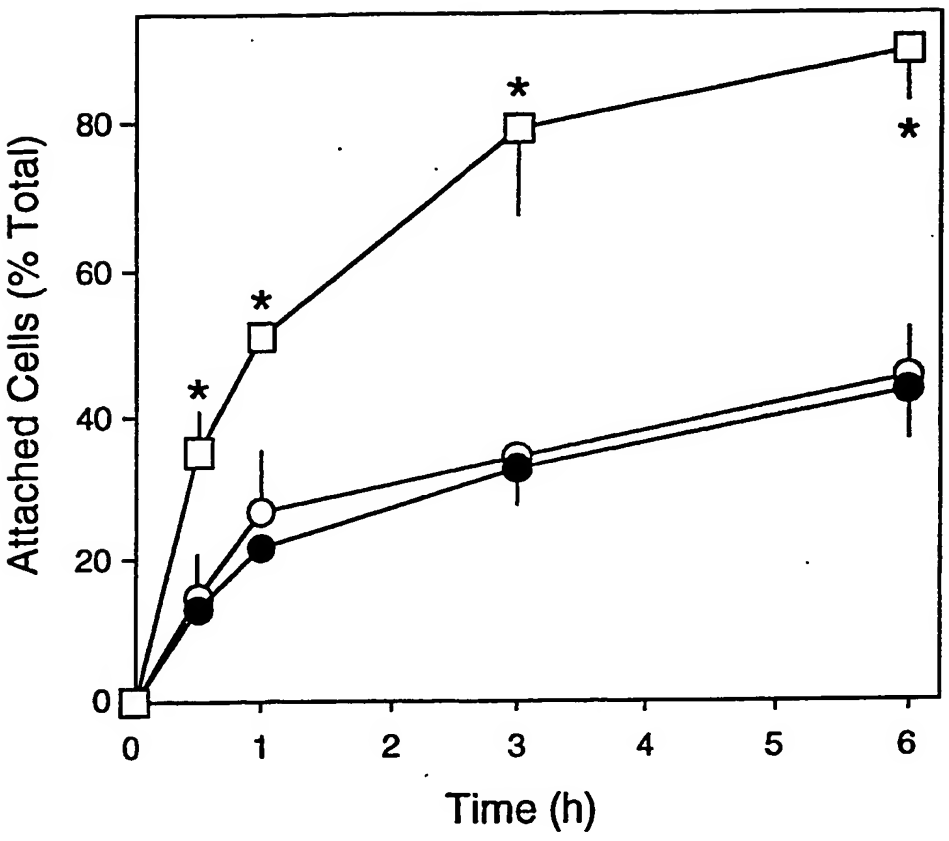

Figure 11

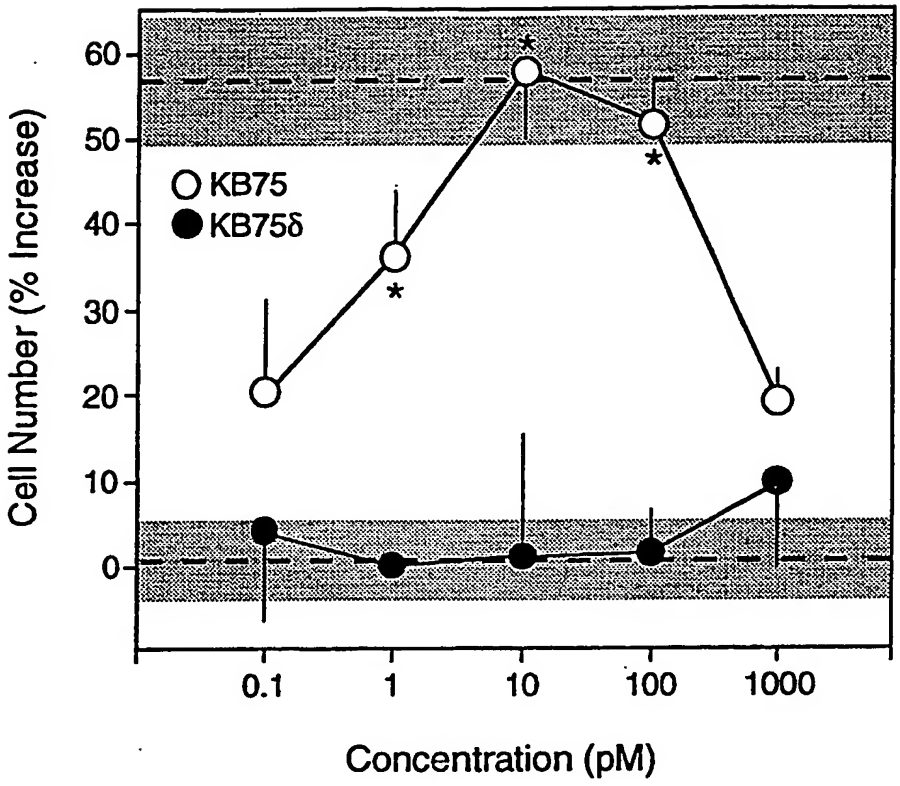
Cell Number (% Increase)
60
50
40
30
20
10
0
KB75
KB75δ
*
*
*
0.1
1
10
100
1000
Concentration (pM)

Figure 12

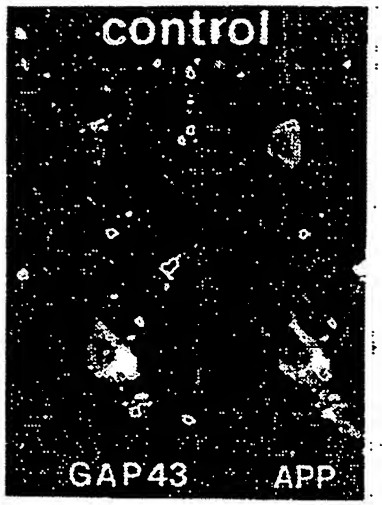

FIGURE 13A1

FIGURE 13A2

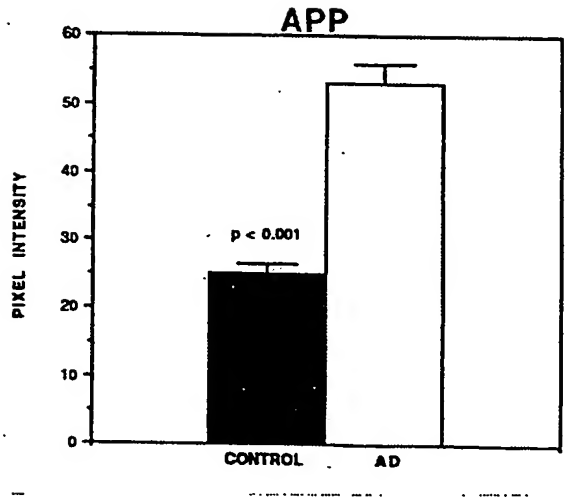

FIGURE 13B

FIGURE 13C1

FIGURE 13C2

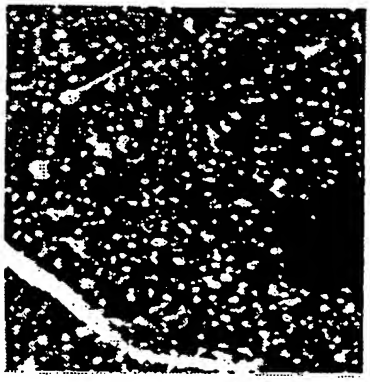

FIGURE 13D1

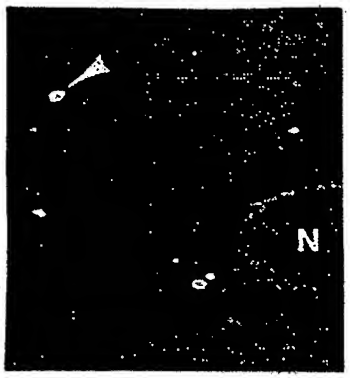

FIGURE 13D2

FIGURE 14A1

FIGURE 14A2

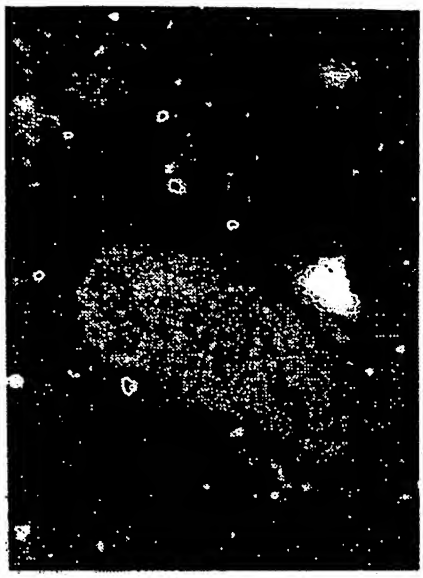

FIGURE 14B1

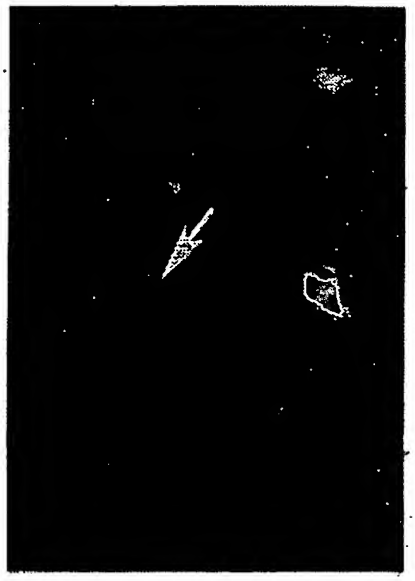

FIGURE 14B2

Fig. 15A1

Fig. 15A2

Fig. 15A3

Fig. 15A4

Fig. 15A5

Fig. 15A6

Fig. 15A7

Fig. 15A8

Fig. 15A9

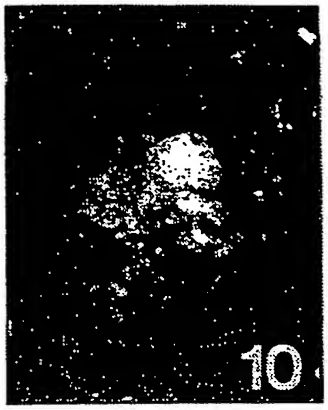

Fig. 15A10

Fig. 15A11

Fig. 15A12

Fig. 15A13

Fig. 15A14

Fig. 15A15

Fig. 15A16

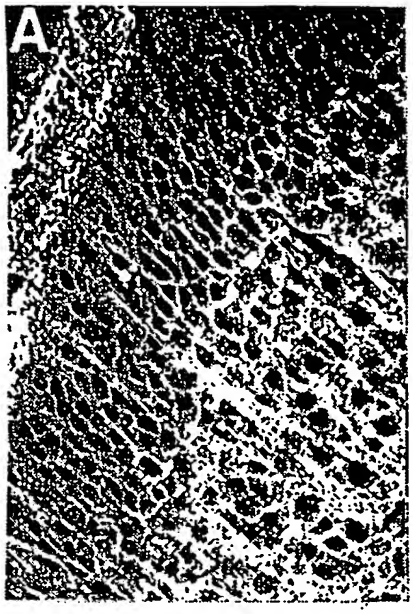

Figure 16A1

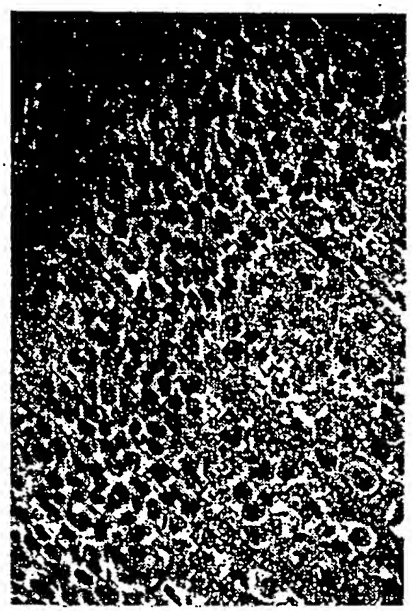

Figure 16A2

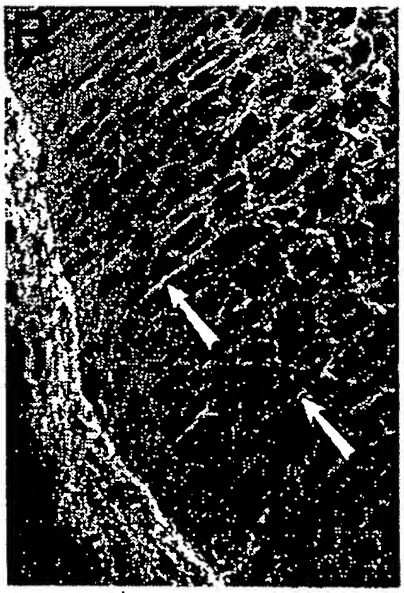

Figure 16B1

Figure 16B2

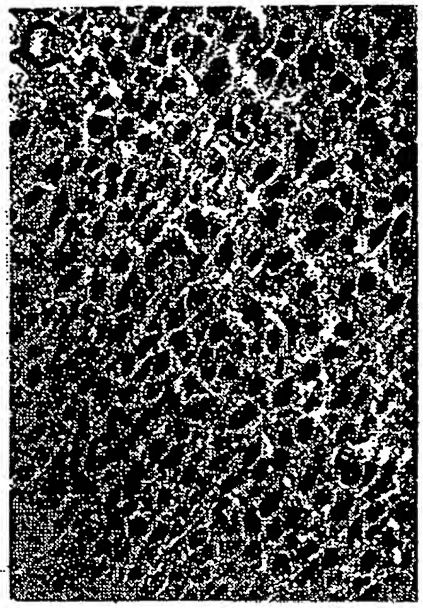

Figure 16C1

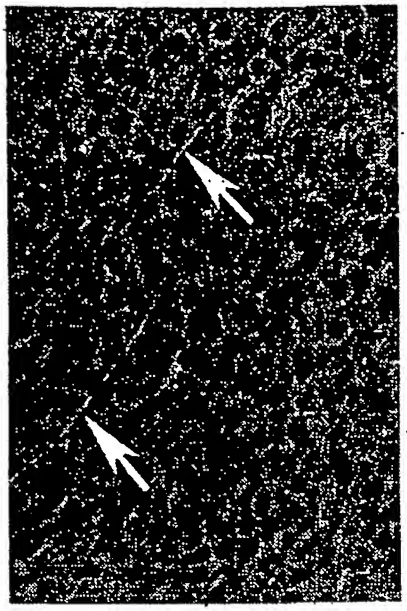

Figure 16C2

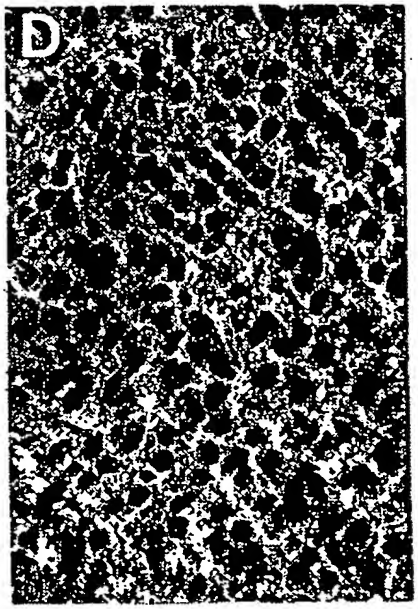

Figure 16D1

Figure 16D2

Fig. 17A1

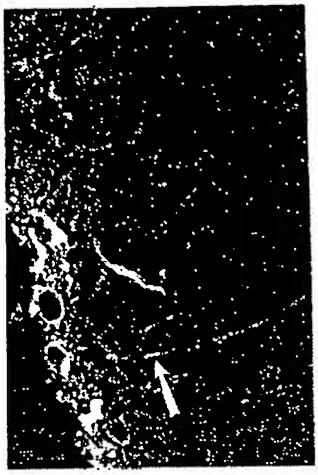

Fig. 17A2

Fig. 17B1

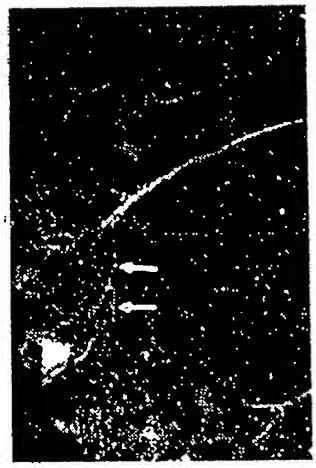

Fig. 17B2

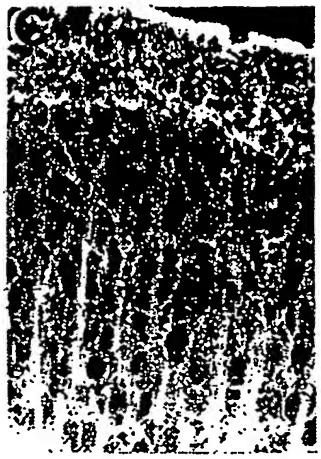

Fig. 17C1

Fig. 17C2

Fig. 17D1

Fig. 17D2

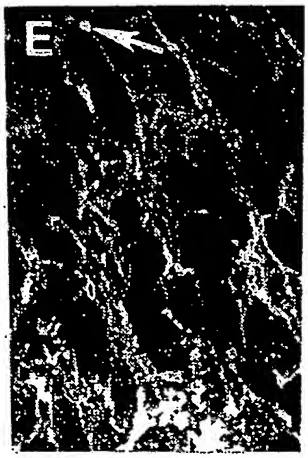

Fig. 17E1

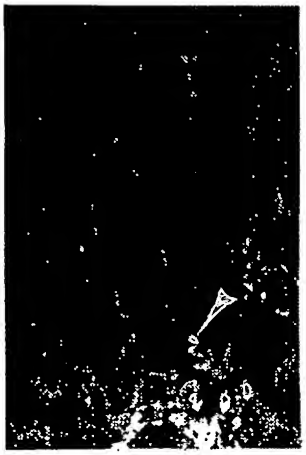

Fig. 17E2

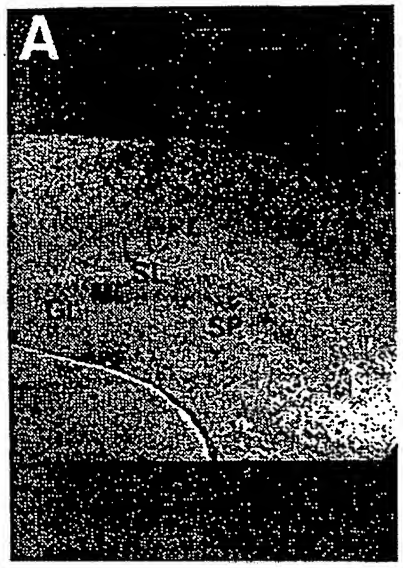

Fig. 18A1

Fig. 18A2

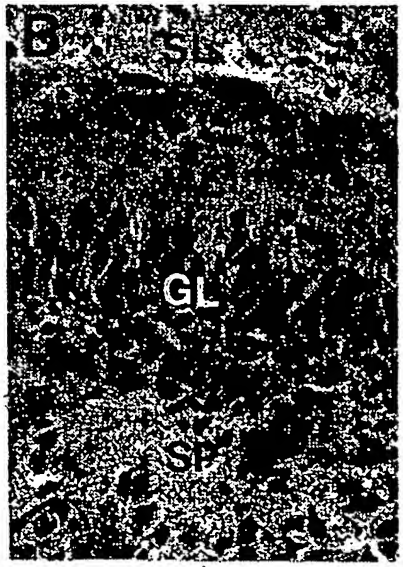

Fig. 18B1

Fig. 18B2

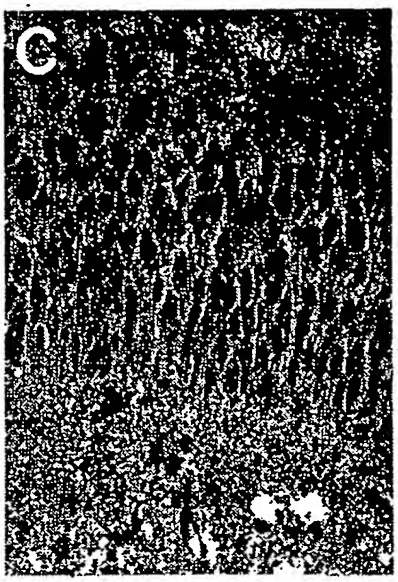

Fig. 18C1

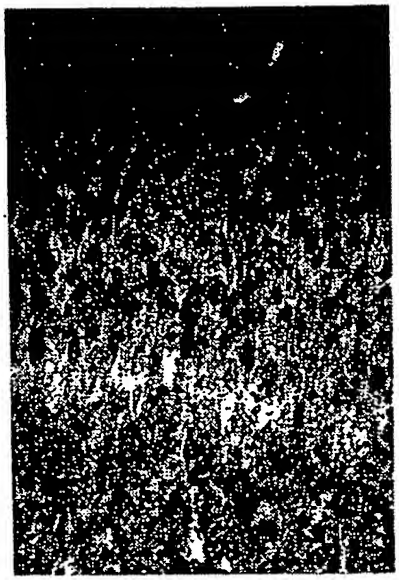

Fig. 18C2

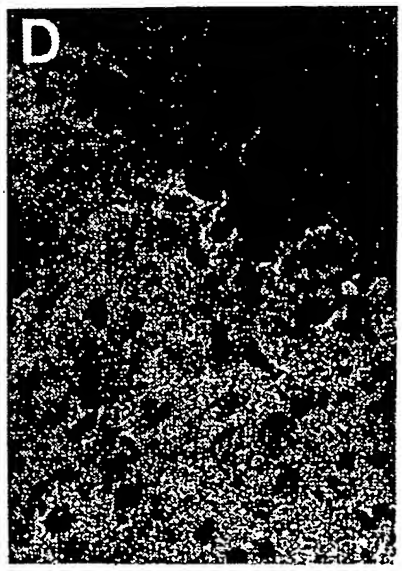

Fig. 18D1

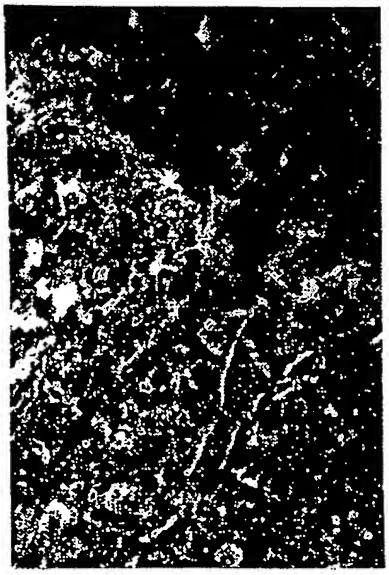

Fig. 18 D2

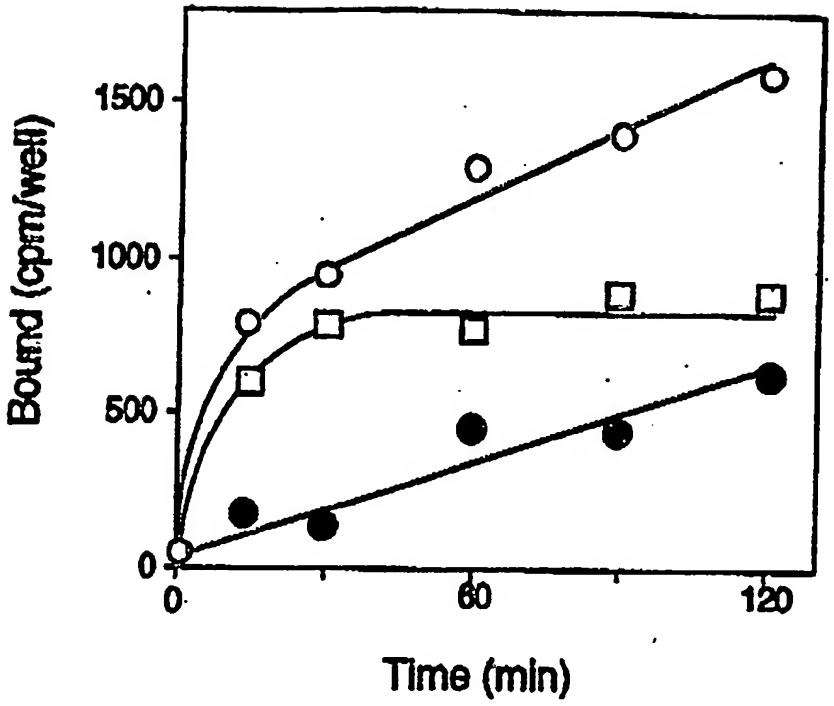

FIGURE 19

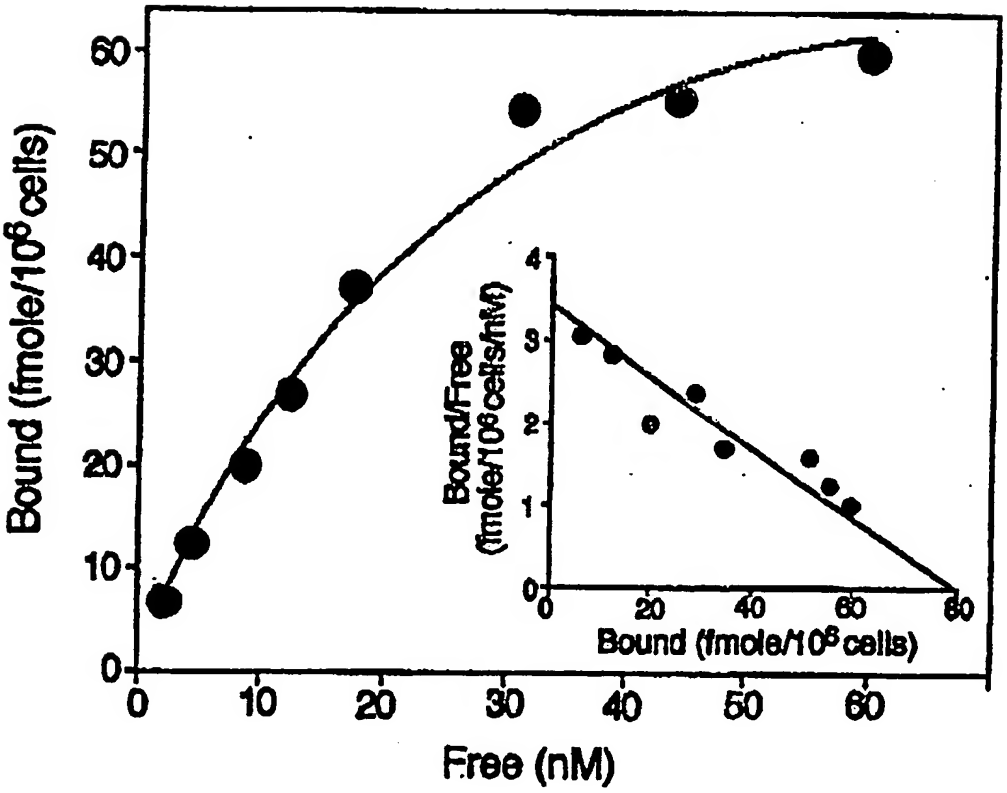

FIGURE 20

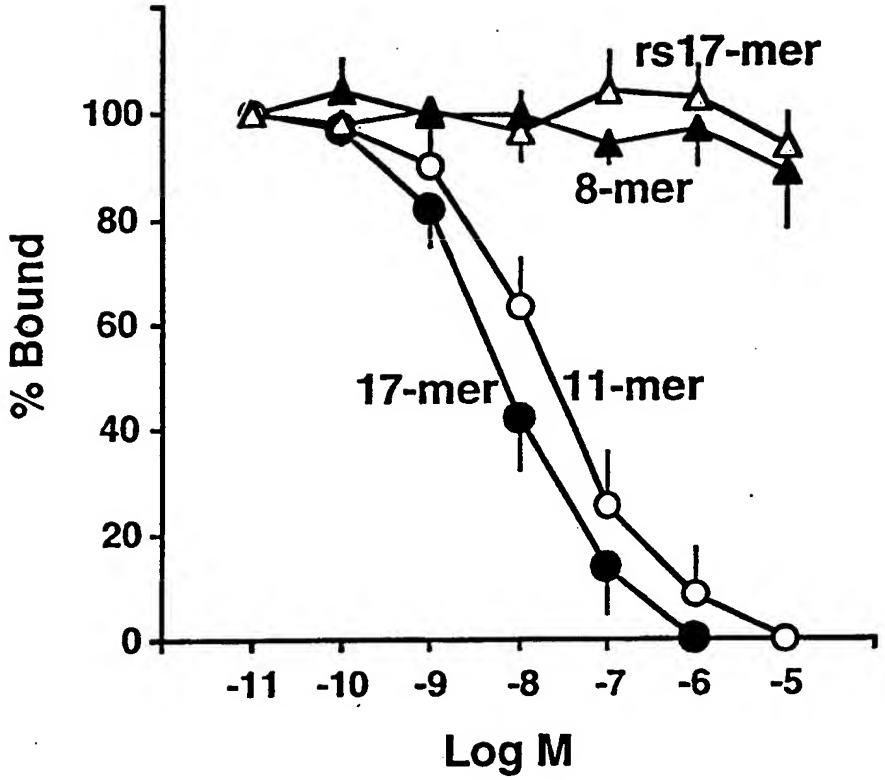
rs17-mer
8-mer
17-mer
11-mer
% Bound
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
Log M

Figure 21

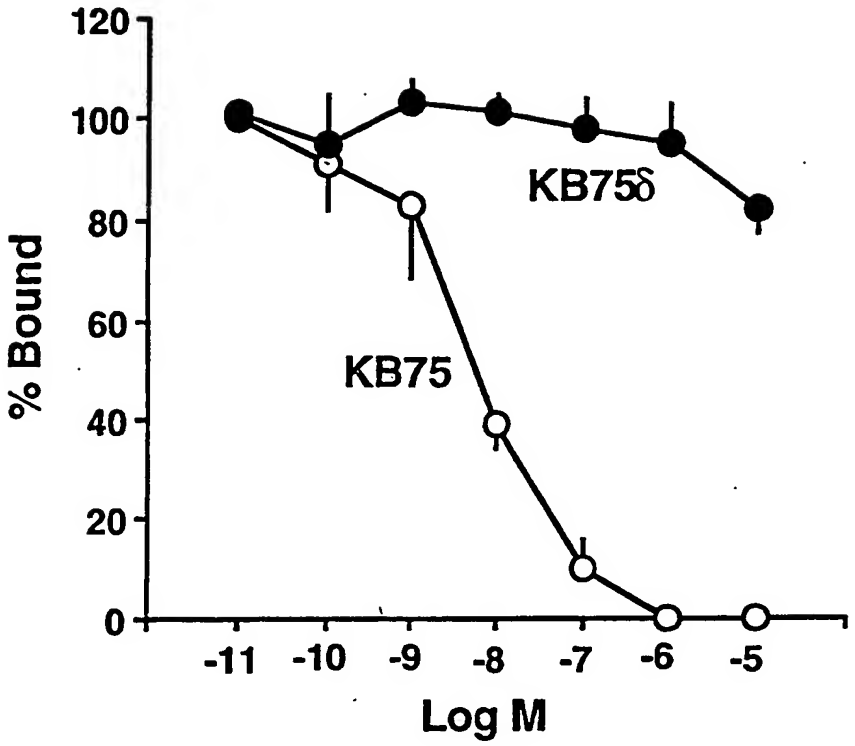

Figure 22

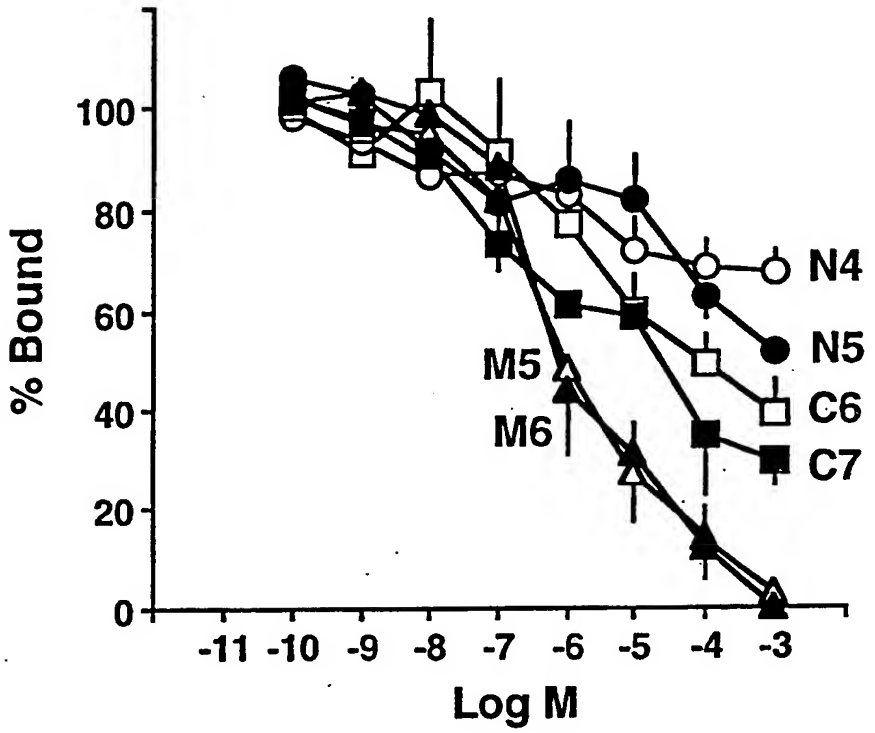

Figure 23

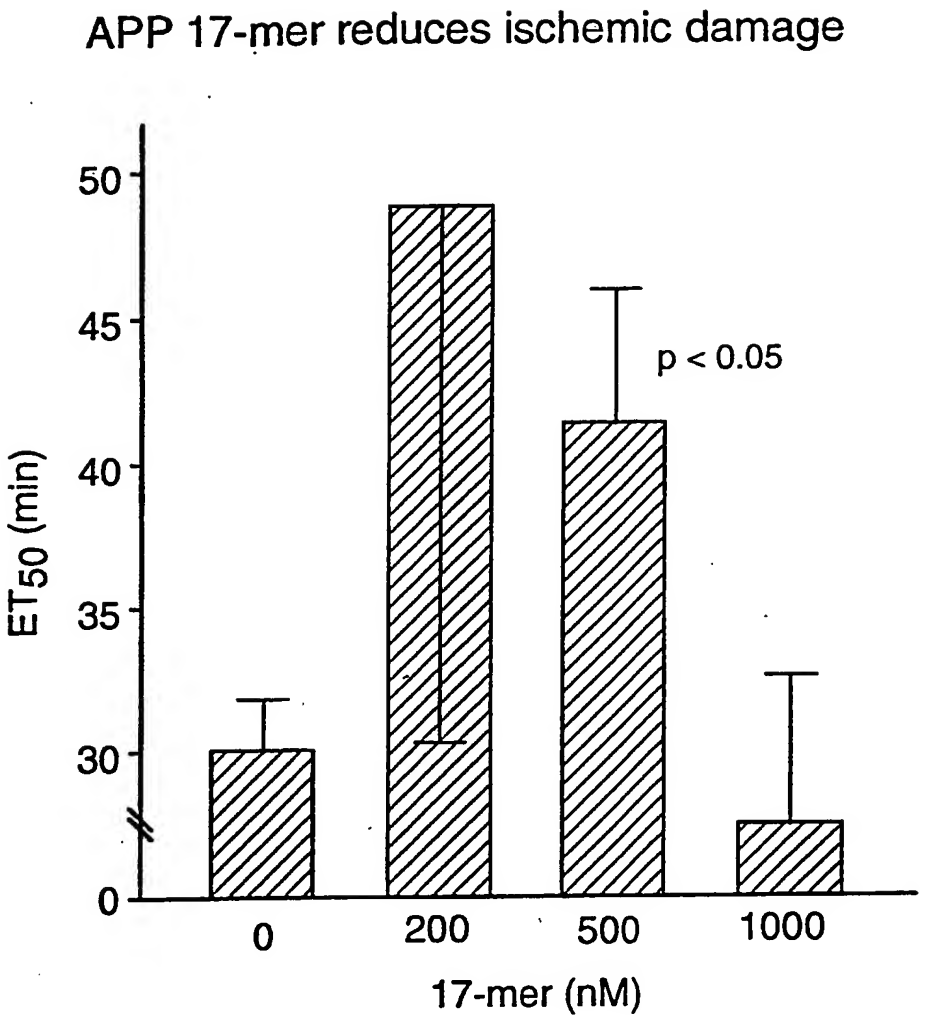
APP 17-mer reduces ischemic damage
50
45
40
35
30
0
ET50 (min)
p < 0.05
0
200
500
1000
17-mer (nM)

Figure 24

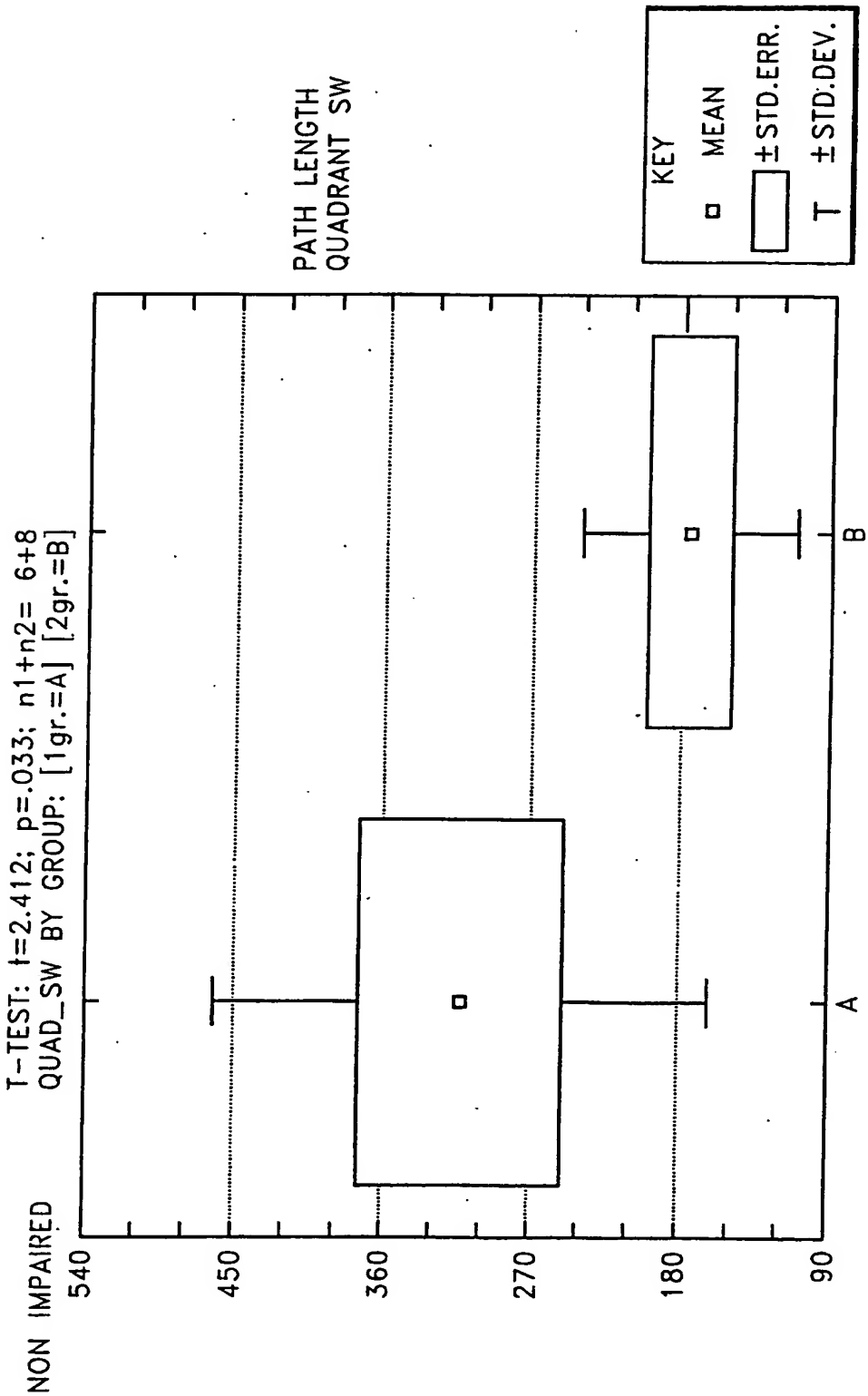
PATH LENGTH
QUADRANT SW
KEY
MEAN
±STD.ERR.
T ±STD.DEV.
T-TEST: t=2.412; p=.033; n1+n2= 6+8
QUAD_SW BY GROUP: [1gr.=A] [2gr.=B]
NON IMPAIRED
540
450
360
270
180
90
A
B

FIG. 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09070

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 5/10, 7/06, 7/08, 7/10, 13/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
Sequences 1-18
amyloid protein precursor, APP, ALZHEIMERS, BRAIN PEPTIDES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A 5,137,873 (YANKNER) 11 AUGUST 1992, SEE ABSTRACT.	1-52
A	US,A 4,963,485 (SHOYAB ET AL.) 16 OCTOBER 1990, SEE ENTIRE DOCUMENT.	1-52
A,E	US,A 5,164,295 (KISILEVSKY ET AL.) 17 NOVEMBER 1992, SEE ABSTRACT.	1-52

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
15 JANUARY 1993	05FEB 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LESTER L. LEE
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09070

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/29; 436/86, 89; 514/12,13,14,15,16,17,18; 530/324,325,326,327,328,329,330,350